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Introduction

One of the central anti-apoptotic pathways in cells is mediated by NF-κB (nuclear factor kappa-B) transcription factors. There is mounting experimental evidence that NF-κB is activated during tumorigenesis in different tissues, and that NF-κB is critically important for cell protection against apoptosis induced by different treatments including chemotherapeutic drugs and γ-irradiation. The key to NF-κB regulation is the inhibitory κB (IκB) proteins which retain NF-κB in an inactive form in the cytoplasm. In response to diverse stimuli, IκBs are rapidly phosphorylated, ubiquitinated and undergo degradation via 26S proteasome. The released NF-κB factors then translocate to the nucleus and activate kB-responsive genes involved in cell growth, apoptosis, and metastasis. Several IκB kinases (IKKs) which trigger IκB degradation and NF-κB activation were discovered in 1997-2000 (Zandi et al., 1997, Peters and Maniatis, 2000). These protein kinases are currently seen as a target of choice to specifically inhibit NF-κB activity. In this project we proposed to test the hypothesis that activation of IKKs and down-stream NF-κB "survival signaling" pathway is a critical event during tumorigenesis in prostate. Our project was focused on the role of IκB kinases IKKα, IKKβ, and IKKε (IKKε is called in the literature also IKKi due to its inducible nature) in constitutive activation of NF-κB in prostate carcinoma (PB) cell lines and PC tumors. We also studied whether IKK blockage induces apoptosis and/or inhibits PC cell growth.

Initially I have received DOD funding for three years: from 05.01.2001 to 04.30.2004. In 2003 my laboratory moved from AMC Cancer Center in Denver to Northwestern University in Chicago. During several months of transition period in 2003 I had to rebuild my laboratory, transfer awards to NU, hire new postdoctoral fellows and technicians; prepare new IRB and IACUC protocols. I have accomplished all these goals successfully. However, it took me some time to rebuild the laboratory, and I had requested and was granted the no-cost extension of the grant till 04.30.2005.

BODY

Research accomplishments.

Aim 1. To determine the role of IkB kinases IKKα, IKKβ, and IKKε in constitutive activation of NF-kB in PC cell lines and PC tumors.

- **Task 1.** Perform analysis of IKKα, IKKβ, and IKKε/i expression in five PC cell lines and primary prostate cell cultures using Northern blotting, Western blotting and immunostaining.
- **Task 2.** Determine IKKα, IKKβ, and IKKε/i function. Determine IKK's phosphorylation. Measure IKK activity in kinase assay; constitutive level of IκB-α phosphorylation and IκB-α stability in five PC cell lines and primary prostate cell cultures.

We successfully completed work proposed in tasks 1 and 2. We have investigated the expression and function of NF-κB, IκB inhibitors and IKKs in normal prostate epithelial cells and prostate carcinoma (PC) cell lines LNCaP, MDA PCa 2b, DU145, PC3, and JCA1. We found that NF-κB was constitutively activated in human androgen-

independent PC cell lines DU145, PC3, JCA1 as well as androgen-independent CL2 cells derived from LNCaP (Gasparian et al., 2002, Fig. 1, p.144). In spite of a strong difference in constitutive κB binding, Western blot analysis did not reveal any significant variance in the expression of NF-kBs (p65 and p50), IκBs, IKKα, and IKKβ between primary prostate cells, androgen-dependent and androgen-independent PC cells (Gasparian et al., 2002, Fig. 3, p. 145 and Fig. 10, p. 147). However, we found that in androgen-independent PC cells IκBα was heavily phosphorylated (Gasparian et al., 2002, Fig. Fig. 5, p. 146), and displayed a faster turnover. To study IκBα turnover in PC cell lines we used several experimental approaches including the direct assessment of the time of IκBα half-life using pulse-chase analysis of metabolically labeled IκBα. We found that IκBα was more than two times stable in LNCaP cells (IκBα half-life was about 60 min) than in DU145 where NF-kB is constitutively active (Gasparian et al., 2002, Fig. 9, p. 147). Using an in vitro kinase assay with IκBα peptide (1-54) that has only Ser32 and Ser36 sites of phosphorylation as a substrate (IKKα/IKKβ complex was immunoprecipitated by treatment of cell lysates with combination of IKKα and IKKβ antibodies) we demonstrated the constitutive activation of IKK in androgen-independent PC cell lines (Gasparian et al., 2002, Fig. 10 B, p. 147).

In contrast to IKKα and IKKβ, IKKε/i was strongly expressed only in androgen-independent PC cells (PC3 and DU145) with high level of constitutively active NF-kB (supplemental Fig. 1). Treatment of PC cells with NF-kB inducers such as IL-1 and TNF-a resulted in a rapid induction of IKKε expression (supplemental Fig. 2). Transient transfection of different PC cell lines with IKKε/i w.t. resulted in the activation of kB.Luciferase reporter, whereas IKKε dominant negative (d.n.) mutant K38A suppressed basal NF-κB activity in PC cells (supplemental Fig. 3). These data provide experimental evidence that IKKε/i could be involved in the regulation of NF-κB activity in PC cells through a positive feedback loop.

We were not able to measure IKKε/i kinase activity because there is no specific substrate for this IKK. The data on IKK expression and activity in PC cells have been published (Gasparian et al., 2002) and presented at the meetings (Gasparian et al., 2001, Gasparian et al., 2002, Yao et al., 2002, Yemelyanov et al., 2003, see appendices # 1, 2, 8, 10).

- **Task 3.** Build a collection of frozen and formalin fixed samples of PC tumors and normal prostate tissues.

We have successfully built the collection of frozen and formalin fixed samples of PC tumor and BPH (benign prostate hyperplasia) samples first via the purchase of prostate samples from the CHTN (NCI cooperative human tissue network, the organization funded by NCI to provide the human tissues for researchers), and later through NU prostate SPORE pathology core. It appeared that normal freshly frozen prostate tissues from organ donors are not available. Because the tissues harvested postmortem could not been used for our studies, we compared in our experiments prostate carcinomas with BPH. Unfortunately the significant part of frozen samples was lost in September 2004 during the accidental power loss in the equipment room, and I had to spend the additional time and effort to get the prostate frozen samples to continue the proposed research.

- **Task 4.** Perform analysis of IKKα, IKKβ, and IKKε/i expression in PC tumors and normal prostate tissues using Northern blotting, Western blotting and immunostaining.

- **Task 5.** Determine IKK function. Measure IKK activity in kinase assay and constitutive level of IκB-a phosphorylation in PC tumors and normal prostate tissues. Determine IKK's phosphorylation.

As proposed we studied the expression of IKK proteins in prostate tissues in parallel with NF-κB proteins. We performed immunostaining of more than 60 formalin-fixed paraffin-embedded samples (including tissue microarrays and individual sections) of BPH, high grade PIN (prostate intraepithelial neoplasia that is considered to be a precancerous lesion), and PCs using multiple antibodies against NF-κB and IKK proteins: p65 (two different Abs from Abcam and Santa Cruz), p50 (Santa Cruz), p52 (Santa Cruz), IKKα (two different Abs from Santa Cruz and Imgenix) , IKKβ (Imgenix), phosphorylated IKKα/ IKKβ (Cell Signaling), and IKKε/i (four different Abs from Imgenix, Santa Cruz., Active Motif, Pro-Sci). We found that there was modest increase of number of p65-positive nuclei in low grade and advanced PCs in comparison to BPH (Gasparian et al., 2002, p.145, Table 1). We did not find significant changes in the expression of p50 (supplemental Fig. 4) in PC in comparison to BPH. There were also no significant changes in the expression of IKKα, IKKβ and IKKε/i in PC in comparison to BPH (supplemental Fig. 5). All studied IKK kinases were expressed in the epithelial compartment of BPH and PCs. Importantly, IKKβ and IKKε/i were mostly found in the cytoplasm of prostate epithelial cells. In contrast, IKKa frequently had nuclear localization (supplemental Fig. 5). Interestingly, P52 expression was higher in high grade PCs in comparison to BPH and low grade PCs. In some tumors p52 immunostaining pattern correlated with IKKα expression, and especially with the level of IKKα/β phosphorylation (supplemental Fig. 6). It is an important finding because IKKα is necessary for processing of p52 precursor p100 (Senftleben et al., 2001). The data on IKKα/β phosphorylation are of special significance. The antibody we used was raised against IKKα/β phosphorylated at Ser176/180. It is known that activation of IKKα and IKKβ requires their phosphorylation at those specific serines in the activation loop of IKK kinases (Karin and Ben-Neriah, 2000). We found for the first time that activated IKKα/β are strongly expressed in the cytoplasm of epithelial cells in PCs (Yemelyanov et al. 2005, Fig. 1). This finding suggests that IKK kinases are indeed constitutively active in prostate tumors, and should be used as a target to inhibit NF-kB. The data on IKKα/β immunostaining have been published (Yemelyanov et al. 2005).

It is known that prostate carcinomas are not localized lesions, thus frozen PC samples contain relatively low number of cancer cells (30-40% at most). This significantly decreases the sensitivity of biochemical approaches including Northern and Western blotting to study NF-kB and IKK expression as well as kinase assay to study IKK activity (see task 5). We were able to isolate good quality RNA from freshly frozen BPH and PC samples. We did not find the significant differences in the expression of IKKα and IKKβ in cancer samples in comparison to BPH samples (supplemental Fig. 7). However, due to the sample nature, the best approach to assess the expression of IKKs in PC is immunostaining.

- **Task 6.** Perform transient transfections of PC3, DU145 and JCA1 PC cells with dominant negative (d.n.) IKKα, IKKβ, and IKKε, kB.Luciferase reporter and RL-null reference construct. Evaluate effect of d.n. IKKs at different doses on the basal and TNF-α-inducible NF-κB activity.

As proposed we studied the role of IKKs in constitutive NF-κB activation in malignant prostate cells using transient transfection of normal prostate epithelial cells and

PC cells with d.n. IKKα (IKKαK44M) or IKKβ (IKKβK44M) mutants. As shown in our publication (Gasparian et al. 2002, Fig. 11, p. 148), both mutants inhibited constitutive luciferase activity in normal and malignant PC3 prostate cells in a similar way, with IKKβ mutant being more potent inhibitor for constitutively active NF-κB. Effect of IKKβ mutant was comparable to the effect of IκBα d.n. mutant (Gasparian et al. 2002, Fig. 11, p. 148). As was described above (see task 2) , IKKε/i dominant negative (d.n.) mutant K38A suppressed basal NF-κB activity in PC cells **(**supplemental Fig.3). To study the effect of d.n. IKK mutants on the inducible NF-kB activity in PC cells we first characterized the sensitivity of PC cells to the individual inducers such tumor necrosis factor alpha (TNF-α), lipopolysaccharide (LPS), and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) using EMSA and transient transfections. We found that despite the high constitutive level of NF-κB activity, androgen independent PC cells appeared to be overall highly sensitive to NF-κB induction (Gasparian et al., 2003, appendix 9 and supplemental table 1). However, the sensitivity of different PC cells to the standard NF-kB inducers differed. For example, DU145 cells are highly responsive to TNF-α and LPS, while PC3 cells are highly responsive to LPS and TPA but not to TNF-α (supplemental table 1). Based on these findings, we selected different NF-κB inducers to study the effect of d.n. IKKβ and IKKα on NF-κB activation in PC3 and DU145 cells. We found that d.n. IKKβ significantly inhibited the NF-kB activation induced by LPS in PC3 cells, and by TNF-a in DU145 cells.

- **Task 7.** Analysis of data. Manuscript preparation.

Aim 2. To determine if IKK blockage induces apoptosis and/or inhibits PC cell growth in vitro and in vivo to address the role of NF-kB signaling pathway in prostate tumorigenesis.

- **Task 8.** Perform stable transfection of PC3 cells with tagged d.n. IKKβ, IKKa, and IKKε/i and empty pcDNA3 vector, select clones with G418. Characterize expression of d.n. IKKs in individual clones and its effect on kinase activity of corresponding IKK. Characterize the effect of d.n. IKKs on the endogenous NF-κB activity in individual clones.

We have generated more than 95 clones of DU145 and more than 80 clones of PC3 cells transfected with d.n. mutants of different IKKs. We have selected positive clones with the highest level of transgene expression and assessed the effect of d.n. IKK mutants on NF-κB function in PC cells using Northern blotting for endogenous kB-responsive genes such as IkBa, and transient transfection with κB.Luciferase reporters. We found that in PC3 clones transfected with IKKβ d.n. mutant and IKKε d.n. mutant constitutive expression of endogenous κB-responsive genes such as IκB-α and IL6 was significantly decreased (supplemental Fig. 8 and Fig. 9). Luciferase assay indicated that both IKKb and IKKε d.n. mutants significantly affected basal and inducible NF-κB activation by selective NF-κB inducers such as LPS and TPA (supplemental Fig. 10). Unfortunately, in most of those transfected clones the expression of d.n. IKKβ, d.n. IKKα, and d.n. IKKε/i mutants significantly decreased during first 10 passages. This did not allow us to use these clones for work proposed in tasks 9-11. To overcome the transgene silencing problem we have undertaken an alternative approach and performed the experiments with highly specific chemical inhibitor of IKKβ, PS1145 (Hideshima et al., 2002)

provided by Millenium Pharmaceuticals Inc. We have developed a comprehensive picture of the effects of PS1145 on NF-kB in PC cells in vitro using EMSA, transient transfections with kB.Luciferase reporters, study of IkBa-phosphorylation, and Northern blot analysis of NF-kB –responsive endogenous genes (Yemelyanov et al., 2005, Figs 2,3,4,5). We found that PS1145 inhibited constitutive and inducible NF-κB activity in androgen-independent PC cells, and blocked the constitutive expression of cytokines, including IL6 (Yemelyanov et al., 2005, Fig. 11).

We extended these studies further and compared the effect of different classes of NF-kB inhibitors including proteasome inhibitors and IKK inhibitors on NF-kB activity and sensitization of PC cells to apoptosis. This work is still in progress. However, the preliminary results indicated that in general proteasomal inhibitors are more effective in both tests than IKK inhibitors.

We also repeated the experiments with forced expression of IKK d.n. mutants using the lentiviral approach. Recently we have finished these experiments and generated pulled DU145 and PC clones with high level of d.n. IKKα, IKKβ, and IKKε/i expression. We have started to characterize these clones to assess the effect of specific IKK mutants on basal and inducible NF-kB activity in PC cells.

- **Task 9.** Determine the proliferation rate of IKK-transfected clones in comparison with the proliferation of vector-transfected clones using cell counting and colorimetric methylthiazol tetrazolium (MTT) assays.

Because the expression of d.n. IKKα, IKKβ, and IKKε/i in most of the generated transfectants significantly decreased during first 10 passages we did not use them to test the effect of IKK on proliferation. Instead we evaluated the effect of chemical inhibitor of IKKβ, PS1145 on proliferation using DU145 cells (Budunova et al., 2004, Yemelyanov et al., 2004, Yemelyanov et al., 2005). The effect of PS1145 on proliferation was assessed by several approaches (Yemelyanov et al., 2005, Fig. 6). MTT test revealed 30-35% decrease in DU145 cell numbers 48-72 hrs after PS1145 treatment. BrdU labeling of DU145 cells confirmed the result of MTT test. The number of BrdU-positive cells (cells in S-phase) was decreased by 32±3.25 % in DU145 cell cultures treated with PS1145 for 72 hrs. The inhibition of proliferation was further confirmed by the decrease of Ki67 protein expression known to be present in cells in G1, S, G2 and M phases but not in G0 phase of the cell cycle. The inhibitory effect of PS1145 on DU145 cell proliferation correlated with the change in cell cycle-related gene expression in DU145 cells (Yemelyanov et al., 2005, Fig. 9). Using RT-PCR we found that PS1145 decreased expression of cyclinD1 and cyclinD2 genes but did not induce significant changes in the expression of cyclinB1 and cyclinB2, and Cdks in PC cells, even though some of those cell cycle-related genes have been previously reported to be down-regulated by NF-κB inhibitors (Yemelyanov et al., 2005, Fig. 9).

- **Task 10.** Determine the effect of d.n. IKK transfection on the constitutive and TNF-α-inducible level of apoptosis using immunoblotting with PARP antibody and TUNEL assay.

For the same reason of instability of transgene expression, we did not use d.n. IKKα, IKKβ, and IKKε/i PC clones to study IKK effect on apoptosis. Instead we evaluated the effect of chemical inhibitor of IKKβ PS1145 on apoptosis in DU145 cells as well on the sensitization of DU145 cells to apoptosis induced by TNF-a (Yemelyanov et al., 2004, Yemelyanov et al., 2005). To evaluate the effect of PS1145 on apoptosis in these cells we

measured caspase3/7 activity, and used Western blot analysis to assess poly-(ADP-ribose) polypeptide (PARP) cleavage. We found that treatment of DU145 cells with PS1145 for 48 hrs resulted in strong activation of caspase 3/7 in a dose dependent manner (Yemelyanov et al., 2005, Fig. 7). Western Blot analysis of caspsase 3/7-dependent PARP cleavage also demonstrated that PS1145 induced apoptosis in DU145 cells 48-72 hr after the beginning of the treatment (Yemelyanov et al., 2005, Fig. 7). Importantly, PS1145 sensitized DU145 cells to TNFα-induced apoptosis (Yemelyanov et al., 2005, Fig. 7). These data are in line with the previous observations that NF-κB protects different cells, including PC cells, against apoptosis induced by TNF-α, and that NF-κB blockage by different genetic approaches results in cell sensitization to TNF-α. As expected, the expression of well-known NF-κB-dependent anti-apoptotic genes such as IAP-1, IAP-2 was significantly decreased in DU145 cells with inhibited NF-κB activity (Yemelyanov et al., 2005, Fig. 9). The similar inhibition of those genes was found in PC3 cells, while we did not find any changes in expression of Bcl-2 and Bax. Our data indicated the role of XIAP-associated factor-1 (XAF1), an antagonist of another inhibitor of caspase3/7, x-IAP. We found that XIAF1 expression was significantly increased in PC cells treated with PS1145 (Yemelyanov et al., 2005, Fig. 9). Unexpectedly, some other anti-apoptotic genes have been activated in PC cells after PS1145 treatment. For example, the expression of genes that encode caspase-8 (FLICE) inhibitory protein FLIP and especially cell death regulator Aven (Peter, 2004) was increased in DU145 cells treated with PS1145 (Yemelyanov et al., 2005, Fig. 9).Aven was recently shown to bind both Bcl-x(L) and the caspase9 regulator Apaf-1, thus inhibiting mitochondrial apoptosis (Chau et al., 2000; Figueroa et al., 2004). Interestingly, the effect of PS1145 on Aven expression in PC3 cells, more resistant to PS1145-induced apoptosis, was more pronounced than in DU145 cells (data not shown). This may potentially explain known higher resistance of PC3 cells to apoptosis induced by NF-κB blockage.

- **Task 11.** Inject vector-transfected or d.n. IKK-transfected PC3 cells (two clones/per construct) subcutaneously into both flanks of athymic nu/nu mice. Totally we will use 40 animals (5 animals/clone, 4 constructs x 2 clones = 8 clones). Monitor bi-weekly tumor growth and animal weight for 6 months. Evaluate percentage of inoculations developing to palpable tumors and growth rate of xenografts.

- **Task 12.** Terminate animal experiment. Harvest tumor tissues. Evaluate the morphology of lesions, expression of transgene (d.n.IKK), NF-κB activity, proliferation index and apoptotic index.

The animal experiment has been postponed until new stable PC3 clones with d.n. IKKs (obtained after PC cell infection with lentiviruses) will be more characterized in vitro. Instead we started the evaluation of the in vivo anti-tumor effect of IKKβ inhibitor PS1145 using the orthotopic intraprostatic injections of PC3 cells transfected with GFP (green fluorescent protein). The usage of GFP-expressing PC3 cells in this experimental model with high frequency of metastatic lesions in the regional lymph nodes should allow us to evaluate the effect of IKKβ inhibition on metastatic capability of PC3 cells using imaging system. In 2003 Dr. Yemelyanov was trained by Dr. W. Bushman at UW School of Medicine to harvest prostate lobes in mice. Thus, we will harvest prostate from mice chronically treated with PS1145. We will specifically focus on the effect of PS1145 on the gene expression in prostate in vivo and will compare the global changes induced

by IKKβ inhibitor in prostate cells in vitro and in vivo using DNA arrays .We have already finished the analysis of PS1145 effect on gene expression in PC3 cells in vitro, and found that inhibition of NF-kB by PS1145 has resulted in significant changes (more than two-fold change, with probability > 0.95) of 167 genes, including genes involved in the regulation of cell cycle, apoptosis and angiogenesis. The results of gene array were validated by quantitative RT-PCR (Yemelyanov et al., 2006, Fig. 9).

- **Task 13.** Analysis of data. Manuscript preparation.

KEY RESEARCH ACCOMPLISHMENTS (KRAs) FY04

- We have analysed the effect of IKK inhibitor PS1145 on gene expression in PC3 and DU145 prostate carcinoma cells using gene arrays. The results were validated by quantitative RT-PCR. Totally, the expression of 176 genes was affected by IKK2 inhibitor PS1145.

- PS1145 inhibited the expression of cell cycle-related genes cyclins D1 and D2, anti-apoptotic genes IAP-1 and IAP-2, and key angiogenesis gene VEGF.

- To counteract the inhibitory effect of PS1145 on NF-κB, PC cells activated the cell survival program and increased the expression of anti-apoptotic genes c-FLIP (inhibitor of caspase 8) and death regulator AVEN.

- Evaluation of the effect of PS1145 on different pro-survival signalling pathways revealed that PS1145 did not affect activity of stress-activated protein kinases SAPK/JNK, inhibited activity of Akt, and activated c-raf/Mek1/2 pathway.

- We compared the effect of two classes of NF-κB inhibitors: proteasome inhibitors (MG132, lactacystin epoxomicin) and IKK inhibitors (PS1145 and Bay 11-7082) on NF-κB activity and apoptosis in PC cells.

- Proteasome inhibitors attenuated NF-κB induction in PC cells more efficiently than IKK inhibitors.

- Proteasome inhibitors were more effective for early sensitization of PC cells to apoptosis induced by TNF-α.

REPORTABLE OUTCOMES FY04

Manuscripts

1. Yemelyanov, A. Gasparian, A., Lindholm, P., Dang, L., Pierce, J., Karsaladze A., and Budunova, I.. Effects of IKK inhibitor PS1145 on NF-kB function, proliferation, apoptosis and invasion activity in prostate carcinoma cells. Oncogene, 2006, 25(3):387-298.

Abstracts presented at the national and international meetings

1. Yemelyanov, A., J. Czwornong, J., Chebotaev, D., Karseladze A., Yang X., Budunova I. Expression and function of glucocorticoid receptor in prostate carcinomas and PC cells. Keystone Symposium: Hormonal regulation of tumorigenesis. February, 2005, Monterey, CA, p. 38.
2. A. Gasparian, N. Gasparian, A. Yemelyanov, D. Chebotaev, F. Kisseljov, and I. Budunova. Targeting NF-κB in prostate carcinoma cells: comparative analysis of proteasome and IKK inhibitors. Keystone Symposium: NF-kB: 20 years on the road from Biochemistry to the patient bed. March -23-28, 2006, Fairmont Banff Springs, Alberta, Canada, accepted.

3. A. Yemelyanov, A. Gasparian, P. Lindholm, L. Dang, F. Kisseljov, A. Karseladze, and I. Budunova. Effects of IKK inhibitor PS1145 on NF-κB function, proliferation, apoptosis and invasion activity in prostate carcinoma cells. Keystone Symposium: NF-kB: 20 years on the road from Biochemistry to the patient bed. March -23-28, 2006, Fairmont Banff Springs, Alberta, Canada, accepted.

Seminar presentations by P.I.

1. Effect of NF-κB inhibitor PS1145 and glucocorticoids on prostate carcinoma cells. Prostate SPORE, R. Lurie Cancer Center, Northwestern University, Chicago, IL, September 2004.

2. Targeting NF-κB transcription factor and IKK kinases in prostate carcinoma cells. The University of Auckland, School of Medicine-Auckland Cancer Society Research Center, Auckland , New Zealand , November 2004.

3. Targeting the transcription factor NF-κB for intervention of prostate and skin cancer. Ludwig Institute for Cancer Research and Royal Melbourne Hospital, Melbourne, Australia, November 2004.

4. Constitutively active NF-κB transcription factor and IKKβ kinase in human prostate carcinoma cells as a possible targets for intervention. Epithelial group seminar series. R.Lurie Cancer Center. Northwestern University. December, 2004.

Conclusions.

IKK inhibitor PS1145 strongly affected global gene expression program in PC cells. As expected IKK inhibitor inhibited the expression of CyclinD1 and CyclinD2, anti-apoptotic genes IAP-1 and IAP-2, and the key angiogenesis gene VEGF. These changes in gene expression correlated well with the inhibition of PC cell growth and sensitization of PC cells to apoptosis induced by TNF-alpha (findings listed in KRAs and in publication Yemelyanov et al., 2006).

Unexpectedly, PS1145 activated a cell survival program. It induced the expression of several anti-apoptotic genes and activated raf/MEK1/2 signaling. We hypothesized that this survival program is induced in PC cells to counteract the effect of NF-kB blockage.

The comparison of two classes of NF-kB inhibitors : proteasome inhibitors and IKK inhibitors revealed that proteasome inhibitors more efficiently inhibit NF-kB and act as more potent sensitizers to apoptosis that IKK inhibitors.

Collaborations for the entire funding period.

As a continuation of the funded research P.I., Dr. Irina Budunova has initiated the collaboration with Millenium Pharmaceuticals Inc. (Cambridge, MA) to study the effect of pharmacological inhibitor of IKKb – PS1145 on PS cell growth, apoptosis and invasion. The results of collaboration are reflected in abstracts (Budunova et al., 2004, Yemelyanov et al., 2004) and in the manuscript (Yemelyanov et al., 1995).

In 2003 P.I. initiated collaboration with Dr. P. Lindholm, an Associate Professor at the Department of Pathology at Northwestern University to study the effect of IKKβ inhibitor PS1145 on PC cell invasion. The results of this collaboration are included in the manuscript published by Yemelyanov et al., Oncogene, 2006 where Dr. Lindholm is a co-author.

In 2003 P.I. became a member of R. Lurie Comprehensive Cancer Center/ Northwestern University Prostate SPORE . In frames of prostate SPORE P.I. is collaborating with Dr. O. Volpert , an Assistant professor at the Department of Urology at Northwestern University to study the effect of NF-kB blockage on the function of androgen receptor. P.I. also started the collaboration with the pathologist Dr. X. Yang, an Associate Professor at the Departments of Pathology and Urology at Northwestern to study the expression of IKKs and steroid hormone receptors in PCs. The results of collaboration are reflected in the new DOD grant submission, where Dr. Yang is a co-P.I..

P.I. continues collaboration with Dr. A. Karseladze, Chair of the Department of Molecular Pathology at N. Blokhin Cancer Research Center (Moscow, Russia) to study the expression of NF-kB and IKKs in PCs. The results of collaboration are reflected in two manuscripts (Gasparian et al., 2002, and Yemelyanov et al., 2005) where Dr. Karsaladze is a co-author.

Promotions

During funded period P.I. has moved from AMC Cancer Research Center in Denver (CO) to Northwestern University in Chicago and was promoted to the position of Assoiciate Professor. Currently P.I. holds the tenure track Associate Professor position at the Department of Dermatology, School of Medicine at Northwestern University (Chicago, IL).

Additional funding obtained/applied based on the work supported by award

1. Working on the proposal supported by this award we made unexpected, exciting and very important findings that urged P.I. to extend the work in the specific direction to study a positive feedback loop in NF-kB activation in PC cells through inducible IKK-related kinase, IKKi. In 2003 the application "Constitutive activation of NF-κB in prostate carcinoma cells through a positive feedback loop: implication of inducible IKK-related kinase (IKKi) was funded by DOD (DAMD17-03-1-0522 , Budunova -PI).

2. In 2004 P.I. initiated in vitro and in vivo studied to evaluate the combined effect of glucocorticoids and IKK inhibitors on PC cell growth. This research "Combinational targeting of NF-kB transcription factor as a novel strategy for apoptosis induction and prostate carcinoma treatment" is supported by developmental project award (to Budunova IV) from Northwestern University Prostate SPORE 5 P50 CA090386-04 (P.I. C. Lee).

3. Looking for the combinational therapeutic approaches to block NF-kB in prostate we became interested in glucocorticoids. Glucocorticoids are very potent inhibitors of NF-kB, and their effect is mediated through negative interaction between glucocorticoid receptor (GR) and NF-kB on protein-protein level. In 2005 P.I. submitted a grant "Role of glucocorticoid receptor in prostate tumorigenesis: from experimental studies to clinical applications" to DOD (Budunova – P.I.). Project PC050636 (idea development award) has not been funded, but has received high priority score, and will be resubmitted after

revision to DOD prostate program in 2006.

The Summary of the reportable outcomes for the entire funding period.

1. Three manuscripts were published in peer-reviewed journals.
2. Eleven presentations were made at the National and International meetings (including two oral presentations at AACR (2001) and Keystone (2005) meetings).
3. Twelve presentations have been given by P.I. at Universities, Research Institutes and Cancer Centers in U.S., Australia and New Zealand.
4. In frames of the supported by DOD research program P.I. has trained three postdoctoral fellows.

Manuscripts (for the entire period).

1. Gasparian, A.V., Yao, Y., Kowalczyk, D., Lyakh, L. A., Karseladze, A., Slaga, T.J.,and Budunova, I.V. Mechanisms of constitutive NF-κB activation in prostate carcinoma cells. Journal of Cell Science, 115: 141-151, 2002.
2. Gasparian, A.V., Yao, Y.J, Lu, J., Slaga T.J, and Budunova, I.V. Selenium compounds inhibit IκB kinase and transcriptional factor NF-κB in prostate cells. Molecular Cancer Therapeutics, Mol Cancer Ther. 1(12):1079-87, 2002.
3. Yemelyanov, A. Gasparian, A., Lindholm, P., Dang, L., Pierce, J., Karsaladze A., and Budunova, I.. Effects of IKK inhibitor PS1145 on NF-kB function, proliferation, apoptosis and invasion activity in prostate carcinoma cells. Oncogene, 2006, 25(3):387-98.

Abstracts presented at national and international meetings (for the entire period).

1. Gasparian, A. V., Yao, Y. J., Lu, J., Slaga, T. J., and Budunova I. V**.** Chemopreventive properties of Selenium compounds are associated with inhibition of IκB kinase and transcriptional factor NF-κB. Proceedings of AACR. 21*supplement:* 78, 2001.
2. Gasparian, A. V., Yao, Y. J., Kowalczyk, D., Slaga, T. J., and Budunova, I. V**.** Mechanism of constitutive NF-κB activation in prostate carcinoma cells. Proceedings of AACR. *21:* 702, 2001.
3. Gasparian, A. V., Yao, Y. J., Kowalczyk, D., Slaga, T. J., and Budunova, I. V. NF-κB is constitutively activated in prostate carcinoma cells. Abstracts of International Symposium: NF-kB regulation, Gene expression and Disease. July 4-8, 2001, Gent, Belgium, p.17.
4. Gasparian A.V., Yao Y.J., Lü J., Slaga T.J. and Budunova I.V. Selenium compounds inhibit IκB kinase and transcriptional factor NF-κB in prostate cancer cells. Keystone Symposium: NF-kB: bench to bedside. February 25-March 3, 2002, Keystone, Colorado, p. 60.
5. Gasparian, A. V., Yemelyanov A.Yu., Yao, Y. J., Kowalczyk, D., and Budunova, I. V. Prostate carcinoma cells secrete a soluble factor(s) responsible for NF-kB activation. Proceedings of AACR. *43:* 364, 2002.
6. Yao, Y. J., Kowalczyk, D., and Budunova, I. V. Role of IKKe in NF-kappaB activation in prostate carcinoma cells. Proceedings of AACR. *43:* 549, 2002.
7. Gasparian, A. V., Yao, Y. J., Slaga T.J. and Budunova, I. V. High sensitivity of prostate carcinoma cell lines to NF-kB induction. Proceedings of AACR, *44*: 1451, 2003.

8. Yemelyanov, A., Yao, Y.J, and Budunova, I. IKKi is a component of the positive feedback loop involved in the constitutive activation of NF-kB in prostate carcinoma cells. Proceedings of AACR *44:* 852, 2003.
9. Yemelyanov A., Gasparian A., Dang L. Pierce J., Budunova I. IKK-beta specific inhibitor PS1145 down-regulates NF-kappaB activity and induces apoptosis in prostate carcinoma cell lines. Keystone Symposium: NF-kB: biology and pathology. January11-16, 2004, Snowbird, Utah, p. 59.
10. Budunova I. , Yemelyanov A., Gasparian A., Dang L., Pierce J. Effect of IKK-beta specific inhibitor PS1145 on NF-kappaB activity and apoptosis in prostate carcinoma cell lines. . Proceedings of AACR *45*, 2004.
11. Yemelyanov, A., J. Czwornong, J., Chebotaev, D., Karseladze A., Yang X., Budunova I. Expression and function of glucocorticoid receptor in prostate carcinomas and PC cells. Keystone Symposium: Hormonal regulation of tumorigenesis. February, 2005, Monterey, CA, p. 38.

Invited talks given by PI (for the entire period).

1. Selenium compounds inhibit IκB kinase and transcriptional factor NF-κB in prostate cancer cells. Chemicon International Inc, Temecula, CA. August, 2001.

2. Selenium compounds inhibit IκB kinase and transcriptional factor NF-κB in prostate cancer cells. Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH. October 2001.

3. Constitutively active NF-κB transcription factor in prostate carcinoma cells as a possible target for intervention. Graduate Program in Cell and Molecular Biology, Colorado State University, Fort Collins, CO. November 2001.

4. NF-κB transcription factor and IKK kinases in prostate carcinoma cells as a possible target for intervention. Grand rounds, Department of Pathology, School of Medicine at the University of Colorado. Denver, CO. November 2001.

5. NF-κB transcription factor and IKK kinases in prostate carcinoma cells as a possible target for intervention. UT M.D. Anderson Cancer Research Center, Department of Carcinogenesis, Smithville, TX. January 2002.

6. Constitutively active NF-κB transcription factor in prostate carcinoma cells as a possible target for intervention. Basic Science Conference, Division of Medical Oncology, University of Colorado Health Sciences Center, Denver, CO. February 2002.

7. Constitutive activation of NF-kB in prostate carcinoma cells: possible role of feedback loops. Prostate Cancer Research Group meeting, UCHSC, Denver, CO, July, 2002.

8. Constitutive activation of NF-kB in prostate carcinoma cells: possible role of feedback loops. Department of Urology, Northwestern University, Chicago, IL, September 2003.

9. Effect of NF-kB inhibitor PS1145 and glucocorticoids on prostate carcinoma cells.

Prostate SPORE, R. Lurie Cancer Center, Northwestern University, Chicago, IL, September 2004.

10. Targeting NF-kB transcription factor and IKK kinases in prostate carcinoma cells. The University of Auckland, School of Medicine-Auckland Cancer Society Research Center, Auckland , New Zealand , November 2004

11. Targeting the transcription factor NF-kB for intervention of prostate and skin cancer. Ludwig Institute for Cancer Research and Royal Melbourne Hospital, Melbourne, Australia, November 2004.

12. Constitutively active NF-κB transcription factor and IKKb kinase in human prostate carcinoma cells as a possible targets for intervention. Epithelial group seminar series. R.Lurie Cancer Center. Northwestern University. December, 2004.

Training of postdoctoral fellows.
In frames of the supported by DOD research program P.I. has trained three postdoctoral fellows:

1. **Dr. Alexander Gasparian**, 1999-2001, at AMC Cancer Research Center, Denver, CO

Current Position: Senior Scientist , National Cancer Research Center, Moscow, Russia, 115478.
Dr. Gasparian is a group leader with independent extramural funding.
Research focus: cell signaling in prostate tumorigenesis, NF-kB –targeted prevention and treatment of prostate cancer

2. **Dr. Alexander Yemelyanov,** 2001-2003, AMC Cancer Research Center, Denver, CO

Current position: Research Associate, Department of Dermatology, NU, Chicago, IL, 60611
Research focus: cell signaling in prostate tumorigenesis, NF-kB – and GR-targeted prevention and treatment of prostate cancer.

3. **Dr. Dmitry Chebotaev**, 2003- present, Department of Dermatology, NU, Chicago, IL. Still in training.

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Appendices

1. Gasparian, A.V., Yao, Y., Kowalczyk, D., Lyakh, L. A., Karseladze, A., Slaga, T.J.,and Budunova, I.V. Mechanisms of constitutive NF-κB activation in prostate carcinoma cells. Journal of Cell Science, 115: 141-151, 2002.
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3. Yemelyanov, A. Gasparian, A., Lindholm, P., Dang, L., Pierce, J., Karsaladze A., and Budunova, I.. Effects of IKK inhibitor PS1145 on NF-kB function, proliferation, apoptosis and invasion activity in prostate carcinoma cells (Oncogene, 2006, 25(3):387-98.
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5. Gasparian, A. V., Yao, Y. J., Kowalczyk, D., Slaga, T. J., and Budunova, I. V. Mechanism of constitutive NF-κB activation in prostate carcinoma cells. Proceedings of AACR. *21:* 702, 2001.
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7. Gasparian, A. V., Yemelyanov A.Yu., Yao, Y. J., Kowalczyk, D., and Budunova, I. V. Prostate carcinoma cells secrete a soluble factor(s) responsible for NF-kB activation. Proceedings of AACR. *43:* 364, 2002.
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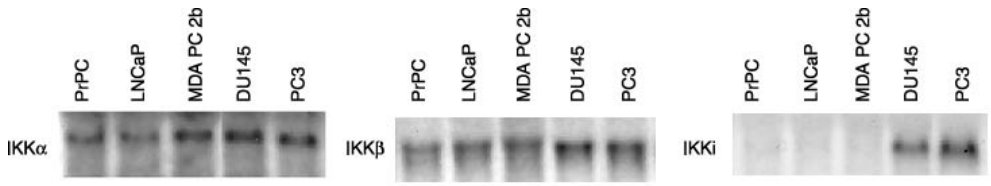
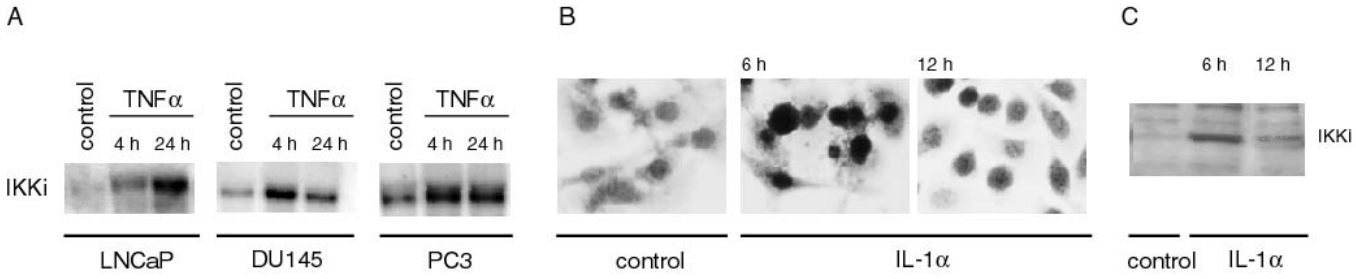
13. Budunova I. , Yemelyanov A., Gasparian A., Dang L., Pierce J. Effect of IKK-beta specific inhibitor PS1145 on NF-kappaB activity and apoptosis in prostate carcinoma cell lines. Proceedings of AACR *45*, 2004 (abstract # 4572).
14. **Supplemental figures 1-10.**
15. **Supplemental table 1.**



Supplemental Fig. 1. Expression of IKKα, IKKβ and IKKε/i in PC cell lines. Northern blot analysis of IKKα, IKKβ and IKKε/i expression in untreated prostate cells. Cells were harvested at 75% confluence and total RNA was isolated and used for Northern blot analysis. Abbreviations for prostate cells: PrEC – normal epithelial prostate primary cultures ,LNCaP and MDA PCa 2b are all androgen-dependent; CL2, JCA1, PC3, and DU145 are androgen-independent PC cell lines.



Supplemental Fig. 2. Induction of IKKε/i in PC cells by cytokines.

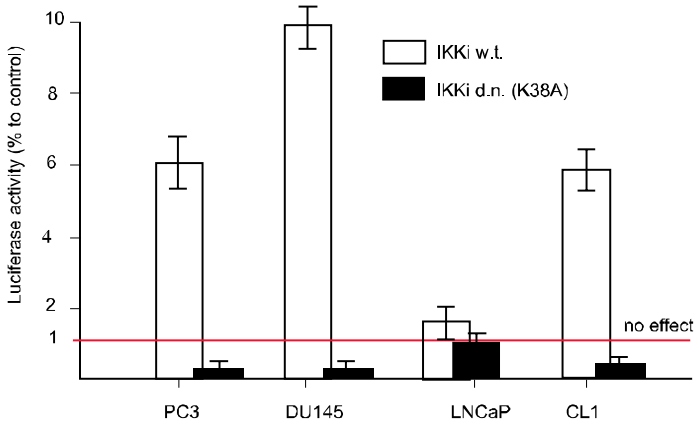
A. Northern blot analysis of IKKi induction in PC cell lines. LNCaP, DU145 and PC3 cells were treated with TNF-α (7.5 ng/ml) and harvested at the indicated time points. Northern blots containing total RNA were probed for expression of IKKi.

B. Analysis of IKKi induction by immunostaining. PC3 cells were treated with IL-1α (7 ng/ml) for indicated time, fixed in cold acetone, blocked with 20% goat serum in PBS, and incubated with primary polyclonal Ab against IKKi (Imgenix).

Note: intensive nuclear IKKi immunostaining in PC3 cells activated with IL-1α for 6 hrs (Fig. 2B, central panel).

C. Western blot analysis of IKKε/i induction in PC3 cells. PC3 cells were treated with IL-1α (7 ng/ml) and harvested at indicated time points. Western blots containing whole cell protein extracts (10 μg/lane) were probed for expression of IKKi using Ab against IKKi (Imgenix).

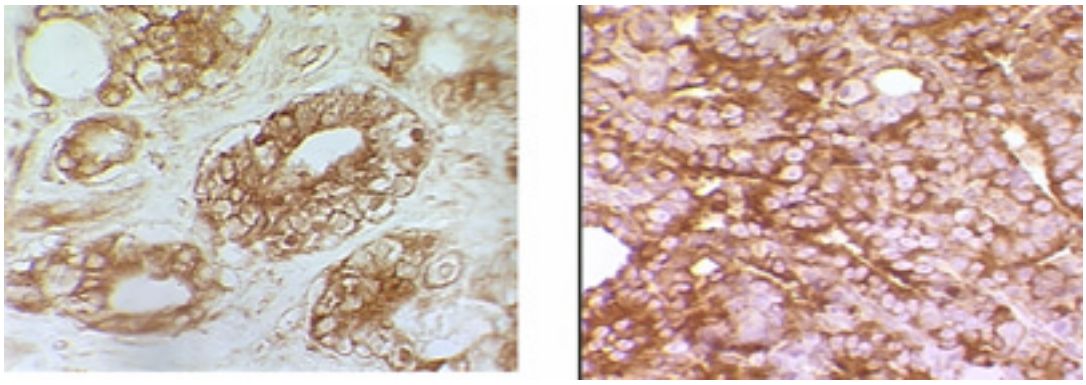
Note: only androgen-independent PC cells express IKKe/i.



Supplemental Figure 3. Effect of IKKε/i d.n. and IKKε/i w.t. on the constitutive activity of kB-luciferase reporter in human PC cell lines. Prostate cells were co-transfected with kB-luciferase reporter (Fireflight luciferase (FL) under promoter with three copies of conventional kB site (Clontech), pRLnull construct - Renila luciferase (RL) under minimal promoter (Promega), IKKe/i d.n. mutant (K38A) or IKKe/i w.t. with Tfx-50 reagent. Luciferase activity was measured 24 hr after transfection in untreated prostate cells by dual luciferase assay. FL activity was normalized against RL activity to equalize for transfection efficacy. The data are presented as fold change compared to the corresponding prostate cells transfected with kB-luciferase reporter and pRLnull only.

Prostate cells descriptions as in the legend for the Fig. 1. CL1- is an LNCaP androgen-independent clone.
Note: IKKi w.t. expression resulted in the increased NF-kB activity in all androgen-independent cells. The expression of IKKi d.n. mutant resulted in the decreased NF-kB activity in all androgen-independent cells.

BPH PC

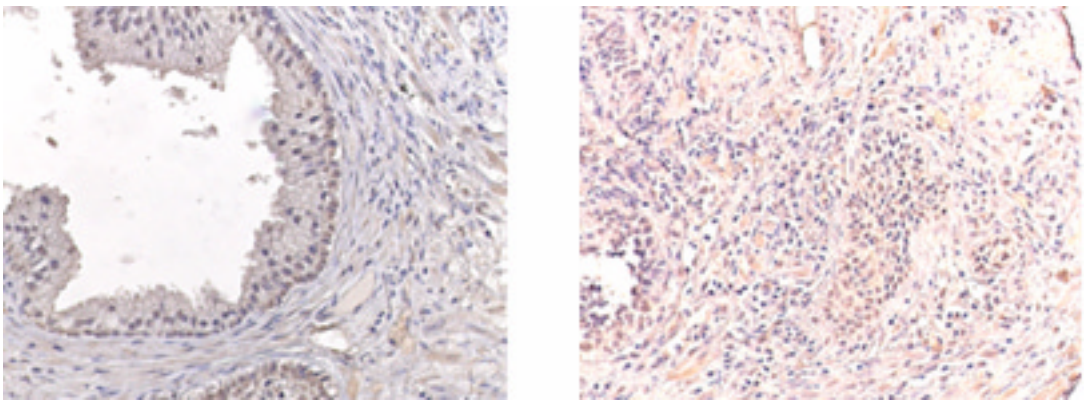


Supplemental Figure 4. p50 expression in BPH and PC tissues.
Formalin fixed paraffin embedded BPH and PC tissues were used for immunostaining. The immunostaining was performed with Envision+ System-HRP (DAB) kit according to the manufacturer's protocol (DakoCytomation, Carpenteria, CA). After Ag retrieval in a pressure cooker in citric buffer (pH 6.0) , tissue sections were blocked with 20% goat serum in PBS, and consequently incubated with primary polyclonal Ab against p50 (Santa Cruz) followed by the secondary anti-rabbit IgG reagent provided with the Envision+ System-HRP (DAB) kit.

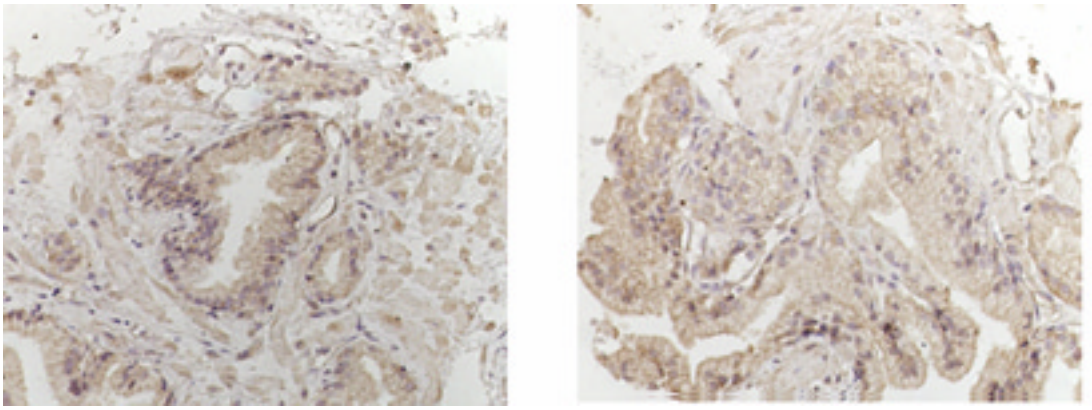
Note: Predominant cytoplasm p50 staining with some positive nuclei in both BPH and PC.

BPH PC

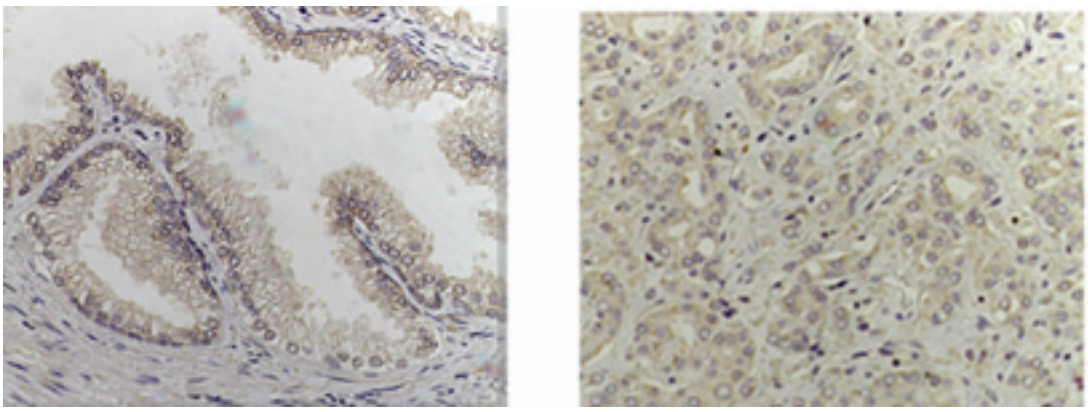
IKKα



IKKβ

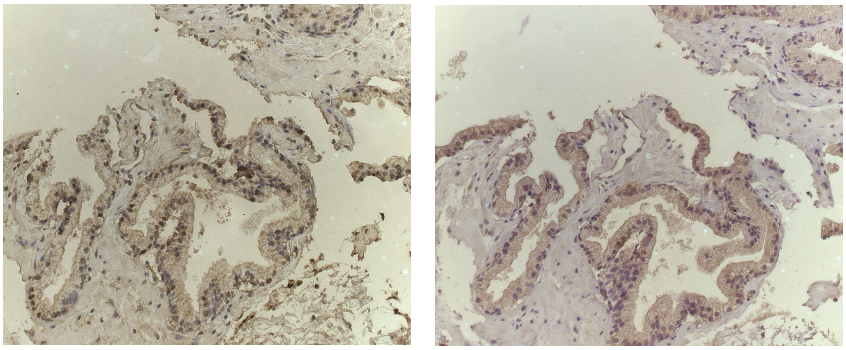


IKKε/i

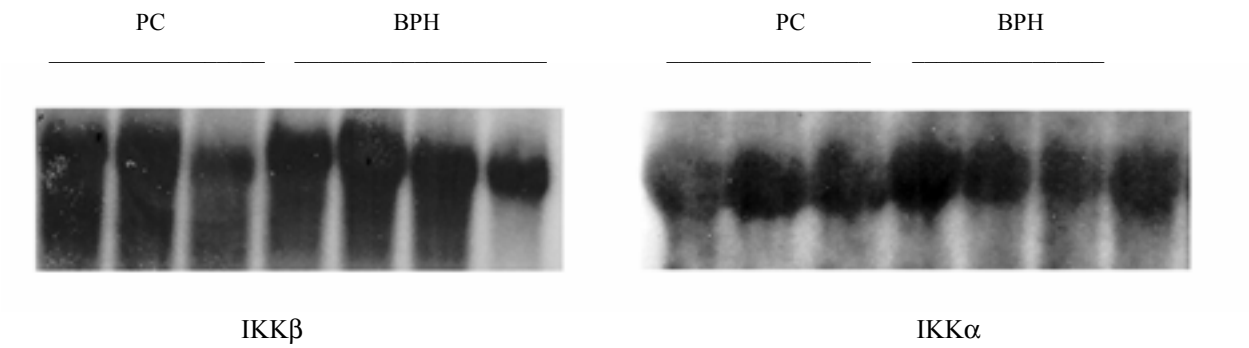


Supplemental Figure 5 . IKK expression in BPH and PC tissues

Formalin fixed paraffin embedded tissues were used for immunostaining. The immunostaining was performed with Envision+ System-HRP (DAB) kit according to the manufacturer's protocol (DakoCytomation, Carpenteria, CA). After Ag retrieval in a pressuire cooker in citric buffer (pH 6.0) , tissue sections were blocked with 20% goat serum in PBS, and consequently incubated with primary polyclonal Ab against IKKα Ab (Santa), IKKβ (Imgenix), and IKKe/i (Imgenix) followed by the secondary anti-rabbit IgG reagent provided with the Envision+ System-HRP (DAB) kit.

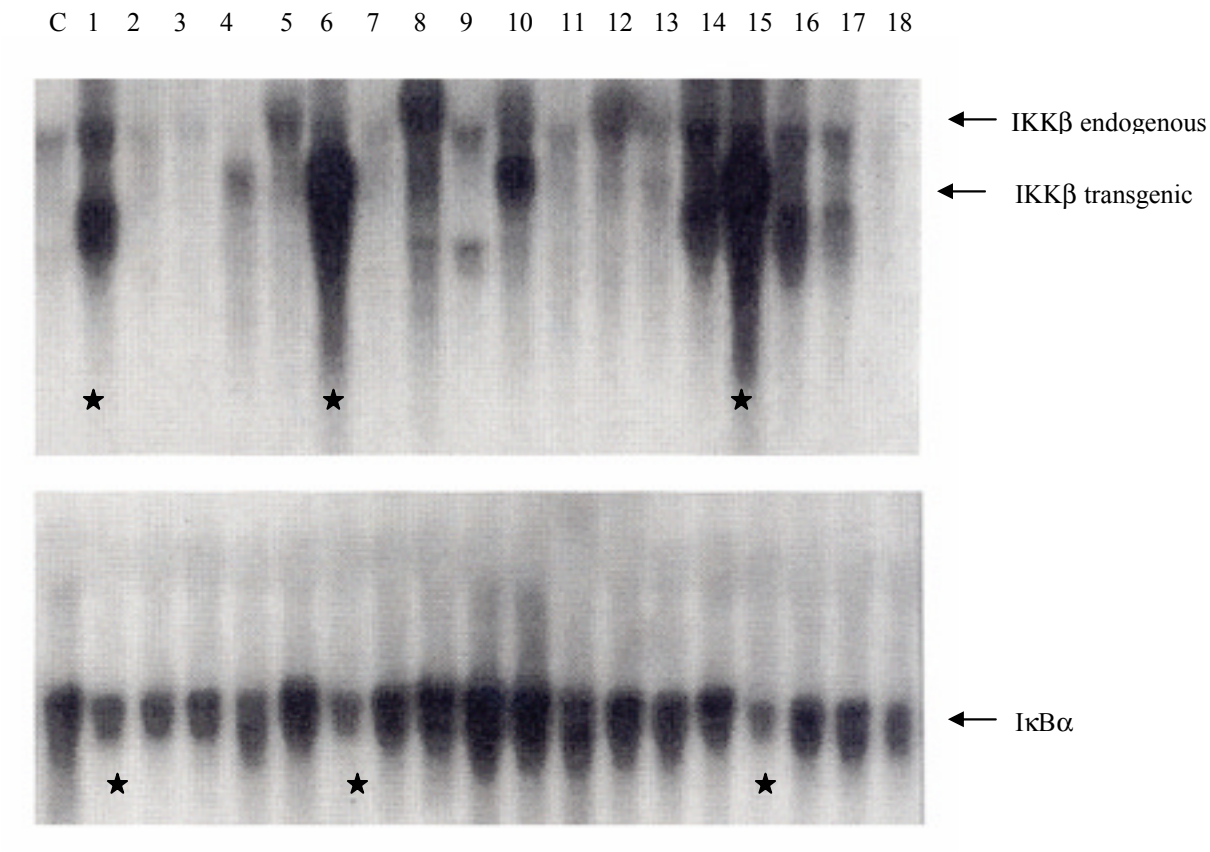


Supplemental Figure 6 . IKKα and p52 expression in PC tissues
Serial sections of formalin fixed paraffin embedded tissues were used for immunostaining. The immunostaining was performed with Envision+ System-HRP (DAB) kit according to the manufacturer's protocol (DakoCytomation, Carpenteria, CA). After Ag retrieval in a pressure cooker in citric buffer (pH 6.0) , tissue sections were blocked with 20% goat serum in PBS, and consequently incubated with primary polyclonal Ab against IKKα Ab (Santa Cruz) or p52 (Santa Cruz) followed by the secondary anti-rabbit IgG reagent provided with the Envision+ System-HRP (DAB) kit.
Note: the similar pattern of localization of p52 and IKKα in prostate carcinoma.



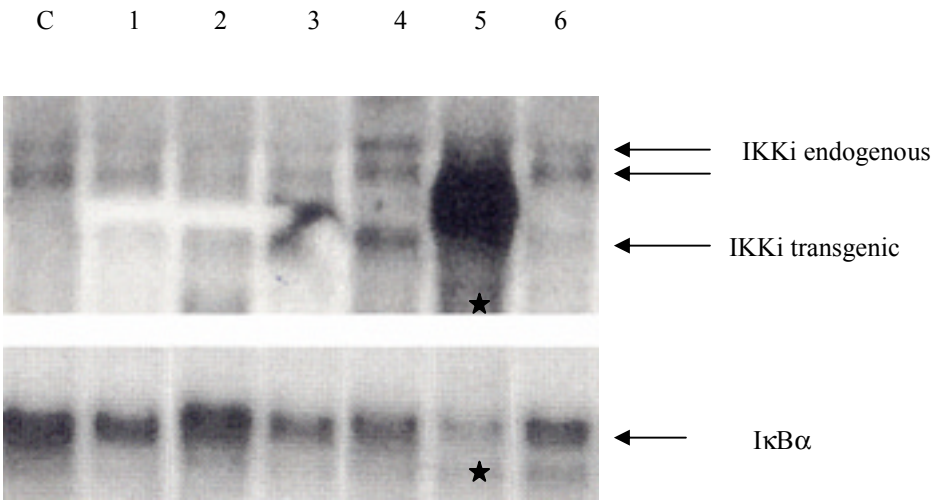
Supplemental Figure 7. Northern blot analysis of IKKα and IKK β expression in BPH and PCs.

Total RNA was isolated from freshly frozen BPH and PC samples (samples were obtained from prostate SPORE core) using TRI reagent (Molecular Research Center) and subjected to Northern blotting. RNA was loaded at 30 μg/lane, resolved in 1% agarose-6% formaldehyde gel, tranferred to nilon membranes and probed for IKKα and IKKβ. The DNA probes were prepared by random-primed reactions using the complete coding sequence of human for IKKα and IKKβ cDNAs (ATCC). Samples 1-3 – PCs; samples 4-7 – BPH.



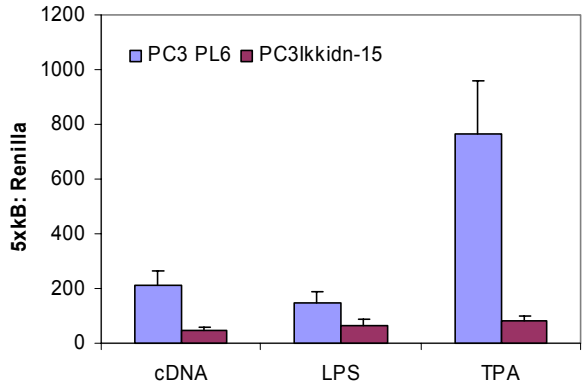
Supplemental Figure 8. Northern blot analysis of IKKβ d.n. mutant stable expression in PC3 clones and its effect on IκBα expression.

PC3 cells were stably transfected with IKKβ d.n. mutant under CMV promoter. After selection with antibiotic G418, eighteen IKKβ d.n clones and several vector transfected PC3 cells (C) were grown to 70% confluency. Cells were harvested. RNA was isolated as described in Fig. 7. The expression level of the mutant was assessed by Northern blotting using the complete coding sequence of human IKKβ cDNA (ATCC). To assess the effect of IKKβ on IκBα expression, the same membrane was reprobed with complete coding sequence of human IκBα .
Note that the clones with highest level of IKKb d.n. expression (clones # 1, 6, and 15 marked by arrows) express low level of IκBα mRNA.



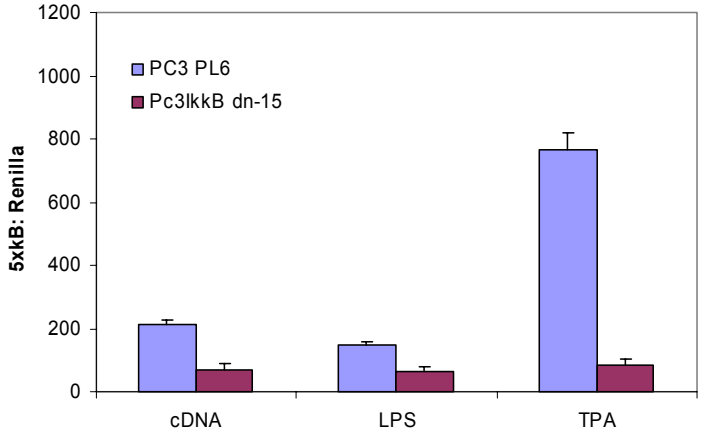
Supplemental Figure 9. Northern blot analysis of IKKε/i d.n. mutant expression in PC3 cells and its effect on IκBα expression.

PC3 cells were stably transfected with IKKε d.n. mutant under CMV promoter. After selection with antibiotic G418, six IKKε d.n clones and several vector transfected PC3 cells (C) were grown to 70% confluency. Cells were harvested, and RNA was isolated as described in Fig. 7. The expression level of the mutant was assessed by Northern blotting using the complete coding sequence of human IKKε cDNA (ATCC). To assess the effect of IKKε on IκBα expression, the same membrane was reprobed with complete coding sequence of human IκBα . Note that the clone with highest level of IKKε d.n. expression (clone # 5 marked by arrows) expresses low level of IκBα mRNA.



A

NF-kappaB PC3 cell line stably expressing IKKi d.n.



B

NF-kappaB PC3 cell line stably expressing IKKbeta d.n.

Supplemental Figure 10 . Effect of IKKε/i d.n. and IKKβ d.n. on the constitutive and inducible activity of kB-luciferase reporter in PC cells.
PC3 cells were stably transfected with IKKε/i (A) and IKKβ d.n. (B) mutants. Clones with the highest level of transgene expression (see Figs 8 and 9) were used for transient transfections. PC3 cells were co-transfected with kB-luciferase reporter (Fireflight luciferase (FL) under promoter with three copies of conventional kB site (Clontech), pRLnull construct (Renila luciferase (RL) under minimal promoter (Promega) with Tfx-50 reagent. 24 hrs after transfection, cells were treated with LPS (1 µg/ml) or TNFα (7 ng/ml) for the additional 24 hrs. In "cDNA group" cells were treated with vehicle only, and used to study the trangene effect on basal NF-kB activity. Luciferase activity was measured in prostate cells by dual luciferase assay. FL activity was normalized against RL activity to equalize for transfection efficacy. The data are presented as fold change compared to control PC3 cells transfected with empty vector (PL6 cells).

Note: both IKKε/i and IKKβ d.n. mutants strongly inhibited basal and inducible NF-kB activity.

Supplemental Table 1. NF-kB induction in PC cells with low and high basal NF-kB activity.

CELL LINE	BASAL κB BINDING **	κB BINDING INDUCTION ***		
		TNF-α	TPA	LPS
PrEC*	Low	+++	+++	+++
LNCaP	Low	+++	++	-
MDA PCa 2b	Low	+++	+++	+++
DU145	High	+++	-	+++
PC3	High	+	+++	++

* PrEC – normal prostate epithelial cells

** Basal kB binding activity was evaluated by EMSA (as described in Gasparian et al., 2002, Fig. 1). Inducible kB binding was evaluated by EMSA in cells treated with TNF-a (7.5 ng/ml), TPA (1 μg/ml) or LPS (1 μg/ml) for 30-60 min.

The role of IKK in constitutive activation of NF-κB transcription factor in prostate carcinoma cells

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Summary

Rel/NF-κB transcription factors are implicated in the control of cell proliferation, apoptosis and transformation. The key to NF-κB regulation is the inhibitory IκB proteins. During response to diverse stimuli, IκBs are rapidly phosphorylated by IκB kinases (IKKs), ubiquitinated and undergo degradation. We have investigated the expression and function of NF-κB, IκB inhibitors and IKKs in normal prostate epithelial cells and prostate carcinoma (PC) cell lines LNCaP, MDA PCa 2b, DU145, PC3, and JCA1. We found that NF-κB was constitutively activated in human androgen-independent PC cell lines DU145, PC3, JCA1 as well as androgen-independent CL2 cells derived from LNCaP. In spite of a strong difference in constitutive κB binding, Western blot analysis did not reveal any significant variance in the expression of p50, p65, IκBs, IKKα, and IKKβ between primary prostate cells, androgen-dependent and androgen-independent PC cells. However, we found that in androgen-independent PC cells IκBα was heavily phosphorylated and displayed a faster turnover. Using an in vitro kinase assay we demonstrated constitutive activation of IKK in androgen-independent PC cell lines. Blockage of NF-κB activity in PC cells by dominant-negative IκBα resulted in increased constitutive and TNF-α-induced apoptosis. Our data suggest that increased IKK activation leads to the constitutive activation of NF-κB 'survival signaling' pathway in androgen-independent PC cells. This may be important for the support of their androgen-independent status and growth advantage.

Key words: NF-κB, IκBα phosphorylation, IKK, Prostate cancer

Introduction

The signaling pathways that regulate cell proliferation, survival and transformation are of prime interest in cancer biology. Recently, the Rel/NF-κB transcription factors, the known regulators of immune and inflammatory responses, have been found to be critically important for control of cell proliferation, apoptosis and tumor development (Rayet and Gelinas, 1999). The Rel/NF-κB transcription factors are homo- and heterodimers consisting of proteins from the Rel/NF-κB family. In mammals the Rel/NF-κB family includes five proteins: NF-κB1 (p50/105), NF-κB2 (p52/100), RelA (p65), RelB and c-Rel. In unstimulated cells NF-κB is sequestered in the cytoplasm by inhibitory molecules, IκBα, IκBβ, IκBε, as well as precursors of NF-κB1 and NF-κB2, proteins p105 and p100. Most agents that activate NF-κB employ a common pathway based on the phosphorylation of the two N-terminal serine's in IκBs, with subsequent ubiquitination and degradation of these proteins by the 26S proteasome (Whiteside and Israel, 1997; Heissmeyer et al., 1999). The released NF-κB factors then translocate to the nucleus and activate transcription of κB-responsive genes. Signal-induced phosphorylation of IκBs is executed by a 900 kDa complex called 'signalosome' containing two inducible IκB kinases IKKα and IKKβ, as well as several structural proteins (Zandi et al., 1997; Yamaoka et al., 1998)

Several lines of evidence suggest that aberrant NF-κB regulation is associated with oncogenesis in mammalian systems. Amplification, overexpression or rearrangement of all genes coding for Rel/NF-κB factors with exception of RelB have been found in leukemias and lymphomas (Rayet and Gelinas, 1999). Constitutive activation of NF-κB is a common characteristic of many cell lines from hematopoietic and solid tumors (Rayet and Gelinas, 1999; Baldwin, 1996; Wang et al., 1999; Dejardin et al., 1999; Bours et al., 1994; Nakshatri et al., 1997; Sovak et al., 1997; Visconti et al., 1997, Palayoor et al., 1999; Duffey et al., 1999; Barkett and Gilmore 1999). The blockage of NF-κB activity in carcinoma cell lines by different approaches dramatically reduced their ability to form colonies in agar and reduced their growth in vitro and in vivo (Visconti et al., 1997; Duffey et al., 1999). It is important that NF-κB also plays a key role in cell protection against diverse apoptotic stimuli including chemotherapeutic drugs and γ-irradiation through activation of the anti-apoptotic gene program in cells (Barkett and Gilmore, 1999).

In spite of the growing evidence of the important role of NF-κB in tumorigenesis and resistance to chemotherapy, only a few attempts have been made to analyze the mechanisms of constitutive activation of NF-κB in transformed cells. It was found that mechanisms involved in NF-κB activation in tumor cell lines could be different, and include increased expression of NF-κB proteins, especially p50 and p52, mutations and deletions in IκBα gene and increased IκBα turnover

(Devalaraja et al., 1999; Krappmann et al., 1999; Budunova et al., 1999; Rayet and Gelinas, 1999).

The aim of this study was to develop a comprehensive and detailed picture of changes in basal NF-κB activity in a panel of prostate cells including primary prostate epithelial cells and six prostate carcinoma (PC) cell lines, and to elucidate the molecular mechanisms that could account for the NF-κB activation in PC cells, including the level of expression of Rel/NF-κB proteins, mutations in the IκBα gene, and IκBα turnover. Our results indicate that NF-κB is constitutively activated in human androgen-independent PC cells. We did not reveal any significant differences in the expression of various NF-κB and IκB proteins or IκBα mutations in any of the examined cell lines. Instead, in androgen-independent PC cells IκBα was heavily phosphorylated and displayed a shorter half-life. Our results indicate that aberrant IKK activation in androgen-independent PC cells leads to the constitutive activation of NF-κB 'survival signaling' pathway, possibly contributing to their growth advantage.

Materials and Methods

Cell cultures and treatments

LNCaP, MDA PCa 2b, DU145 and PC3 cells were purchased from American Type Culture Collection (Rockville, MD), JCA1 cells (Muraki et al., 1990) were received from O. Rokhlin (University of Iowa, Iowa City, IA). The androgen-independent CL2 cells derived from LNCaP cells via an in vitro androgen deprivation, were received from A. Belldegrun (Jonsson Comprehensive Cancer Center, University of California, Los Angeles, CA). Primary prostate epithelial cells were purchased from Clonetics Corporation (Walkersville, MD). LNCaP, CL2, DU145, PC3 and JCA1 were cultured in RPMI 1640 medium (Gibco BRL Life Technologies, Rockville, MD) supplemented with 10% FBS (HyClone, Logan, UT), 1 mM sodium pyruvate (Sigma Chemical Co., St Louis, MO), 0.1 mM β-mercaptoethanol (Sigma) and antibiotics. Primary prostate epithelial cells and MDA PCa 2b were cultured in the media and under conditions recommended by ATCC and Clonetics Corporation accordingly. Cells at 80% confluency were treated with 10 μg/ml cycloheximide (CHX) (Biomol Research Laboratories, Inc., Plymouth, PA), 7.5 μg/ml MG132 (Biomol Research Laboratories Inc.) or 3.2 μg/ml 15-deoxy-Δ^{12-14}-prostaglandin J2 (Cayman Chemical Company, Ann Arbor, MI).

Preparation of cellular extracts and electrophoretic mobility shift and supershift assays (EMSA and EMSSA)

Nuclear and cytosolic proteins were isolated as described previously (Lyakh et al., 2000). The binding reaction for EMSA contained 10 mM Hepes (pH 7.5), 80 mM KCl, 1 mM EDTA, 1 mM EGTA, 6% glycerol, 0.5 μg of poly(dI-dC), 0.5 μg of sonicated salmon sperm DNA, [γ-^{32}P]-labeled (2-3×10^5 cpm) double-stranded κB-consensus oligonucleotide (Promega Corp., Madison, WI), [γ-^{32}P]-labeled (2-3×10^5 cpm) double-stranded oligonucleotide representing Sp1-consensus binding site (Santa Cruz Biotechnology, Santa Cruz, CA), and 5-10 μg of the nuclear extract. DNA-binding reaction was performed at room temperature for 30-45 minutes in a final volume of 20 μl. For EMSSA antibodies against p65 (sc-109X), p50 (sc-114X), p52 (sc-298X), c-Rel (sc-71X) or RelB (sc-226X), were added 30 minutes after the beginning of reaction, and incubation was continued for an additional 30-45 minutes. All antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). DNA-protein complexes were analyzed on 6% DNA retardation gels (Novex, Carlsbad, CA). Dried gels were subjected to radiography.

Western blot analysis

Proteins were resolved by electrophoresis on 10-12.5% SDS-PAAGs and transferred to Immobilon-P membrane (Millipore Corporation, Bedford, MA). Polyclonal anti-p50 (# 06-886), anti-p52 (# 06-413) anti-c-Rel (# 06-421) antibodies were from Upstate Biotechnology (Lake Placid, NY). Anti-p65 (sc-372), anti-RelB (sc-226) anti-IκBα (sc-371), anti-IκBβ (sc-946), anti-IκBε (sc-7156) or anti-IKKα/β (sc-7607) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Ser32 IκBα Ab was from Cell Signaling Technology Inc. (Beverly, MA). Anti-PARP Ab was from PharMingen (San Diego, CA). Membranes were blocked with 5% nonfat milk in TBST buffer and incubated with primary antibodies for 1.5 hours at room temperature. Anti-Phospho-Ser32 IκBα Ab required 6 hours incubation at 34°C. Peroxidase-conjugated anti-rabbit IgG (Sigma) was used as a secondary antibody. ECL Western blotting detection kit (Amersham Pharmacia Biotech, Sweden) was used for protein detection. The membranes were also stained with Ponceau Red to verify that equal amounts of proteins were loaded and transferred.

Pulse-chase analysis of IκBα degradation

Metabolic labeling of cells was performed as described previously (Krappmann et al., 1999). Protein extracts were prepared at the indicated time points. Cells were washed twice with cold PBS and lysed in TNT buffer (20 mM Tris, pH 8.0, 200 mM NaCl, 1% Triton X-100) with protease inhibitors as described previously (Lyakh et al., 2000). Lysates were incubated on ice for 15 minutes and then centrifuged for 5 minutes at 13,000 ***g***. Supernatant was used for immunoprecipitation. Immunoprecipitation of 400 μg of the total protein in 3 ml of TNT buffer was performed using IκBαN (#1309) antiserum (a generous gift from N. Rice, NCI, Frederick, MD). Two hours later 20 μl of protein A-sepharose 4B (Sigma Chemical Co.) in TNT buffer were added to each sample and incubated with gentle rotation overnight. Then sepharose beads were washed 5 times with ice-cold TNT buffer and boiled for 5 minutes in SDS-loading buffer. The supernatant was resolved by SDS-PAAG followed by transfer to Immobilon-P membrane (Millipore Corporation).

In vitro IKK activity assay

Unstimulated prostate cells and LNCaP cells treated with TNF-α (7.5 ng/ml) were lysed in TNT buffer with protease inhibitors. Immunoprecipitation of 450 μg of total protein was performed with 1 μl of rabbit IKKα (#1997) and IKKβ (#4137) antisera (a kind gift of N. R. Rice, NCI, Frederick, MD), as described for pulse-chaise assay. Immunoprecipitate was washed three times in TNT buffer with protease inhibitors and twice with kinase buffer without protease inhibitors. Kinase reaction was performed in kinase buffer (20 mM Hepes, pH 7.4, 2 mM $MgCl_2$, 2 mM $MnCl_2$), containing 2 μCi of [γ-^{32}P]ATP and IκBα peptide (1-54) that has only Ser32 and Ser36 sites of phosphorylation (Boston Biologicals Inc., Boston, MA) as a substrate for 30 minutes at 30°C. Then 2× Tricine/SDS sample buffer (Novex, Carlsbad, CA) was added to each reaction, samples were boiled and subjected to PAAG on 10-20% gradient tricine PAAG (Novex). Gels were dried and exposed to film with an intensifying screen at –70°C.

IκBα cDNA sequencing

IκBα cDNA was obtained by RT-PCR from total RNA using previously described primers and conditions (Emmerich et al., 1999) except the modification in sense primer in the fourth pare of primers. We used the primer: 5′-GCTCAGGAGCCCTGTAATGGCC-GGACTG-3′. PCR products were resolved on 1.5% agarose gel, extracted by QIAquick gel extraction kit (Qiagen Inc., Valencia, CA) and subjected to direct sequencing.

Transfection of cell lines and luciferase activity

Prostate cells were plated on 35 mm dishes and at 50% confluence were co-transfected by Tfx-50 reagent (Promega Corp.) with the following constructs: κB-luciferase reporter – *Fireflight* luciferase (FL) under promoter with three copies of conventional κB site (Clontech Laboratories Inc., Palo Alto, CA); pRL-null construct – *Renilla* luciferase (RL) under minimal promoter (Promega); MMTV.luciferase reporter – *Fireflight* luciferase (FL) under control of MMTV promoter (Clontech); kinase-inactive mutants of either IKKα (IKKαK44M) or IKKβ (IKKβK44M) which work in dominant-negative (d.n.) fashion; and IκBα d.n. mutant. Plasmids with IKK mutants were described earlier (Mercurio et al., 1997) and kindly provided by F. Mercurio (Signal Pharmaceutical Inc., San Diego, CA). Plasmid with the IκBα d.n. mutant lacking serine 32 and serine 36 (Van Antwerp et al., 1996) was a kind gift of I. Verma (Salk Institute, San Diego, CA). Tfx-50 reagent (2.25 μl/μg of plasmid DNA) and the plasmid DNAs (all at a dose of 2 μg/dish) κB.Luc, pRL-null, IKKα d.n., IKKβ d.n., and IκBα d.n. were added to the dishes in antibiotic-free, serum-free medium. 24 hours after transfection, prostate cells were harvested in the lysis buffer and the luciferase activity was measured by dual luciferase assay (Promega) as recommended by the manufacturer. FL activity was normalized against RL activity to equalize for transfection efficacy.

Northern blot analysis

Total RNA from freshly harvested cells was isolated by TRI reagent (Molecular Research Center Inc., Cincinnati, OH) and subjected to northern blotting. 20 μg of total RNA was resolved in a 1% agarose/6% formaldehyde gel. The RNA was transferred to nylon membranes and probed for *IκBα* and *IL-6*. The membranes were also hybridized with a 7S RNA probe to verify that equal amounts of RNA were loaded and transferred. The DNA probes were prepared by random-primed reactions using the complete coding sequence of human I*κBα* and *IL-6* cDNAs (ATCC, Rockville, MD).

P65 immunostaining of prostate tumors

Prostate tissues were obtained from white male patients at the age 40-82 years during biopsy or surgery to remove prostate tumors. Paraffin sections of formalin-fixed prostate carcinoma samples with verified diagnosis and surrounding normal tissues were used for immunostaining. After microwave Ag retrieval and blocking with 5% nonfat milk in PBS, tissues were incubated with primary rabbit polyclonal p65 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) followed by secondary biotinylated anti-rabbit IgG. Immunostaining was visualized with streptavidin-alkaline phosphatase/histo mark red reagent (Kirkegaard & Perry, Gaithensburg, MD). Sections were counterstained in Mayer's hematoxylin.

Adenovirus infection and apoptosis detection

Prostate cells were plated on 35 mm dishes and at 50% confluence were infected with type 5 recombinant Adenovirus (AdV) construct AdV-d.n.IκBα encoding green fluorescent protein (GFP) and mutant human IkBα protein with substitution of serines 32 and 36 to alanines (32A36A) or adenovirus encoding only GFP (AdV-control). AdV-d.n.IkBα virus with deletions of E1 and E3 was generated using the AdEasy1 system. AdEasy1 system was a generous gift of T.-C. He (The Howard Hughes Medical Institute, Baltimore, MD) (He et al., 1998). Mutations of IkBα were constructed by site-directed mutagenesis with the Bio-Rad Muta-Gene Phagemid In Vitro Mutagenesis system (Bio-Rad Laboratories, Hercules, CA) as described (Whiteside et al., 1995). IkBα mutant has an N-terminal tag (ADRRIPGTAEENLQK) derived from the Equine Infectious Anemia Virus (EIAV) tat protein. Control E1/E3-deleted AdV 5 with GFP (AdV-control) was purchased from Quantum Biotechnologies (Montreal, QC, Canada). Adenoviruses were purified by CsCl gradient centrifugation. Cells were infected with adenoviruses (10^9 vp/dish) in 700 μl of medium with 0.5% serum overnight. 24 hours after infection cells were treated with 7.5 ng/ml TNF-α (R&D Systems, Minneapolis, MN) for 10 hours. Apoptosis was determined morphologically by counting the number of blebbing cells out of 200 fluorescent cells per slide. In addition, we used PARP proteolysis to determine apoptosis. Adherent cells and detached floaters were combined for whole-cell protein extract preparations. PARP cleavage was estimated by western blot analysis with PARP antibody (PharMingen, San Diego, CA).

Data in all figures are shown as results of the representative experiments. All experiments were repeated at least three times.

Results

NF-κB is constitutively activated in PC androgen-independent cell lines In the present study we compared NF-κB function in primary prostate epithelial cells, and androgen-dependent and androgen-independent PC cell lines, which in a way represent sequential stages of prostate tumor development towards hormone-independent growth.

To evaluate NF-κB DNA-binding activity, we performed an electrophoretic mobility shift assay (EMSA) using nuclear protein extracts. We found a strong increase of κB DNA-binding in androgen-independent DU145, PC3 and JCA1 cell lines compared with normal prostate epithelial cells and androgen-dependent LNCaP and MDA PCa 2b cells (Fig. 1A). It is important to note that κB DNA-binding was higher in androgen-independent CL2 cells derived from androgen-dependent LNCaP cells via an in vitro androgen deprivation (Fig. 1A). Significantly, the level of NF-κB binding in androgen-independent cells was similar to one in LNCaP cells treated with TNF-α (Fig. 1A, last lane). To rule out the general effects that some transcriptional regulators in androgen-independent PC cells have in κB-binding, we performed EMSA with Sp1 oligonucleotide. As shown in Fig. 1C, Sp1 binding activity did not correlate with androgen-dependence of growth. It was equally low in androgen-dependent LNCaP cells, their androgen-independent counterpart CL2, and androgen-independent DU145 cells. By contrast, Sp1 binding was much higher in androgen-dependent MDA PCa 2b cells and in androgen-independent JCA1 cells. Thus, NF-κB was specifically upregulated in androgen-independent PC cells.

Analysis of nuclear κB-binding complexes was done using electrophoretic mobility super shift assay (EMSSA). As shown in Fig. 1B, the incubation of nuclear extracts from PC3 cells with anti-p50 antibody removed both complexes while incubation of extracts with anti-p65 antibody removed only the upper complex. Similar results were obtained by EMSSA for other PC cells (data not shown). Incubation of extracts with anti-p52, anti-c-Rel, and anti-RelB antibodies did not affect complex mobility, although those antibodies properly worked in EMSSA with positive control samples (data not shown). In addition, western blot analysis showed the lack of c-Rel and RelB protein expression in normal and malignant prostate cells (data not shown). Thus, in all studied prostate cells the constitutive complexes were represented by p65/p50 and p50/p50 dimers.

To study NF-κB functional activity we performed transient transfection of primary prostate epithelial cells obtained from

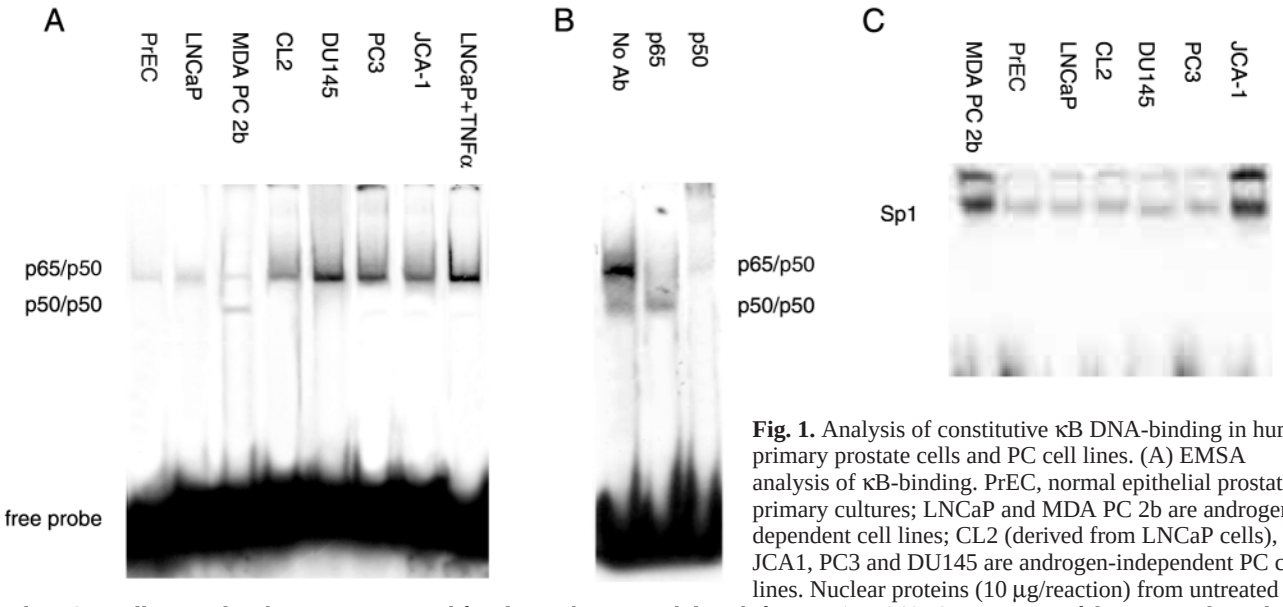

Fig. 1. Analysis of constitutive κB DNA-binding in human primary prostate cells and PC cell lines. (A) EMSA analysis of κB-binding. PrEC, normal epithelial prostate primary cultures; LNCaP and MDA PC 2b are androgen-dependent cell lines; CL2 (derived from LNCaP cells), JCA1, PC3 and DU145 are androgen-independent PC cell lines. Nuclear proteins (10 μg/reaction) from untreated cells and LNCaP cells treated with TNF-α were used for electrophoretic mobility shift assay (EMSA). Composition of dimers is indicated. Data are shown for one representative experiment. (B) Identification of nuclear κB-binding complexes by EMSSA. Nuclear proteins from PC3 cells were incubated with a labeled κB oligonucleotide and antibodies against p50 and p65 proteins. DNA binding activity was analyzed by EMSA. Composition of dimers is indicated. (C) EMSA analysis of Sp1-binding. Nuclear proteins (10 μg/reaction) from the same cells as in Fig. 1A, were used for EMSA with Sp1 oligonucleotide. Composition of dimers is indicated. Data are shown for one representative experiment.

two different donors and several PC cell lines with exogenous κB-responsive gene, κB-luciferase reporter. The results of these experiments presented in Fig. 2A, in general correlated well with the EMSA results: the basal activity of κB reporter was much higher in androgen-independent PC3 and JCA1 cells than in primary prostate cells and LNCaP cells.

We also evaluated the expression of κB-responsive endogenous genes IκBα and IL-6 genes which are tightly regulated by NF-κB in different cells and contain several κB-binding sites in the promoter region (Le Bail et al., 1993; Zhang et al., 1994). As expected, the results of northern blot analysis demonstrated high constitutive levels of IκBα and IL-6 mRNA expression in androgen-independent DU145 and PC3 cells (Fig. 2B). The levels of IκBα and IL-6 mRNA expression in JCA1 cells were comparable with those in androgen-dependent cells possibly due to the absence of some other factors necessary for transcription of these genes in JCA1 cells.

To extend our observation of increased NF-kB activity in PC cells lines, we performed p65 immunostaining of ten samples of human PC obtained during biopsy and two samples of PC with apparently normal surrounding prostate tissues obtained during prostatectomy. The results clearly showed that p65 was overexpressed in the epithelial component of tumors in comparison with the surrounding tissues. Moreover, p65 was localized both in cytoplasm and in the nuclei of cells in PC: 23±8% of nuclei in PC were p65-positive compared to

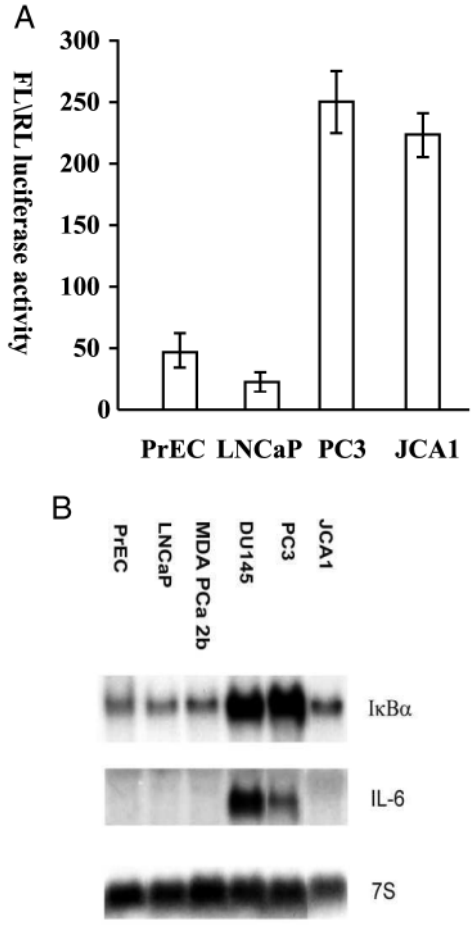

Fig. 2. Analysis of constitutive NF-κB transcriptional activity in human primary prostate cells and PC cell lines. (A) Constitutive activity of κB-luciferase reporter. Prostate cells were cotransfected with κB-luciferase reporter and pRL-null construct. Luciferase activity was measured 24 hours after transfection in untreated prostate cells by dual luciferase assay. Data are shown as FL/RL ratio for one representative experiment. (B) Northern blot analysis of constitutive *IκBα* and *IL-6* genes expression. Northern blots containing total RNA (20 μg/lane) from untreated normal prostate and PC cells were probed for expression of *IκBα* and *IL-6* genes. The membranes were also hybridized with a 7S RNA probe as a control for equal RNA loading.

Table 1. Nuclear expression of p65 in human prostate carcinomas and in apparently normal surrounding prostate tissue

Prostate carcinomas		Normal surrounding prostate	
Sample number	p65 nuclear staining*	Sample number	p65 nuclear staining*
1‡	25%	1	9.5%
2‡	28%	2	10.5%
3	11%		
4	14%		
5	15%		
6	18%		
7	16%		
8	21%		
9	37%		
10	28%		
11	29%		
12	30%		

*The number of p65-positive nuclei is presented as a percentage of 200-300 nuclei evaluated in prostate epithelial cells per section.
‡Samples were obtained during prostatectomy.

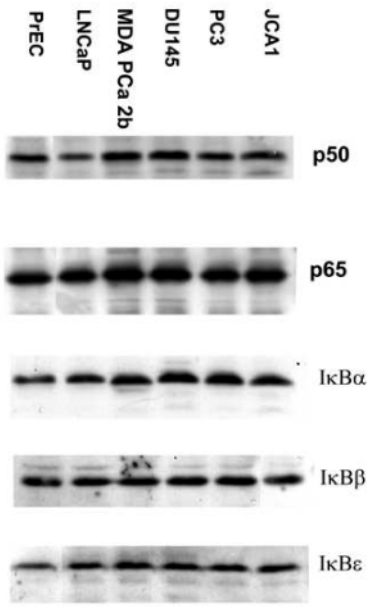

Fig. 3. Western blot analysis of NF-κB and IκB protein expression in human primary prostate cells and PC cell lines. Western blots containing whole cell protein extracts (10 μg/lane) from untreated cells were probed for expression of p50, p65, IκBα, IκBβ and IκBε.

10.5±0.7% of nuclei in normal tissues (Table 1). Translocation of p65 to the nucleus strongly suggests that NF-κB is activated in prostate tumors. Unfortunately hormone-dependence of tumors could not be assessed because we used biopsies and surgically removed PC tissues from untreated patients.

Activation of NF-κB in PC cell lines is not caused by changes in NF-κB and IκB expression or structure

As a first step to elucidate the mechanism(s) leading to the NF-κB activation in androgen-independent PC cells we have analyzed the expression of p50, p65, IκBα, IκBβ and IκBε in comparison with primary prostate cells and androgen-dependent LNCaP and MDA PCa 2b cells. Western blotting of whole-cell protein extracts has not revealed any significant changes in the level of expression of p50, p65 or IκBs in all studied cells (Fig. 3). None of the studied cells expressed RelB or c-Rel (data not shown). The analysis of molecular weights of NF-κB and IκB proteins in PC cells did not reveal any deviations from the expected sizes, suggesting that there were no large alterations of NF-κB and IκB proteins in all studied cell lines.

It was shown that IκBα protein is truncated/mutated in cell lines from some hematopoetic tumors (Rayet and Gelinas, 1999; Emmerich et al., 1999; Cabannes et al., 1999). To address the question whether NF-κB activation in androgen-independent PC cells could be a consequence of mutations or small deletions in the *IκBα* gene, we performed sequencing of IκBα cDNAs obtained by RT-PCR from JCA1, PC3 and DU145 cells. The direct sequencing of IκBα cDNA has not predicted any amino acid substitutions in IκBα protein in those cell lines with constitutive NF-κB activation.

Even though we showed that IκBα is not mutated in PC cells, we could not rule out that other IκB proteins are mutated or functionally impaired in those cells. Thus, in our next set of experiments we addressed the question whether NF-κB activation in androgen-independent PC cell lines is a result of altered interaction between NF-κB and IκB molecules using the universal inhibitor of all IκB degradation, MG132 (Sun and Carpenter, 1998). We expected that MG132, which blocks proteasome-dependent IκB proteolysis, will inhibit basal κB DNA binding if interaction between NF-κB and IκBs in androgen-independent cells is normal. As shown in Fig. 4, MG132 indeed strongly inhibited basal κB DNA binding in PC3 and DU145 cells 30-60 minutes after treatment (Fig. 4). MG132 also decreased κB DNA binding in JCA1 cells 1 hour after treatment (Fig. 4). These results suggest that NF-κB is normally controlled by IκBs in PC cells. Thus, the increased basal NF-κB activity in these cells is not a result of expression of mutated IκB or mutated NF-κB proteins constitutively present in the nucleus.

Increased IκBα phosphorylation and turnover in PC androgen-independent cell lines

Induced NF-κB activation requires IκBα phosphorylation at Ser32 and Ser36 followed by IκBα ubiquitination and degradation (Whiteside et al., 1995; Traenckner et al., 1995). To study IκBα turnover in PC cell lines we used several experimental approaches. First we compared the level of IκBα phosphorylation in different PC cells by western blotting with antibodies directed against IκBα phosphorylated at Ser32.

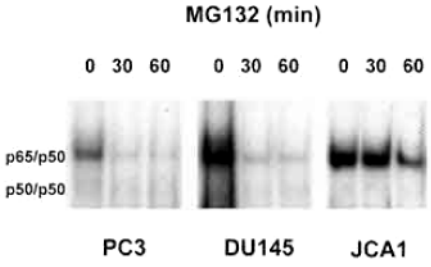

Fig. 4. Proteasomal inhibitor MG132 decreased κB DNA binding in androgen-independent PC cells. Androgen-independent cell lines were treated with proteasomal inhibitor MG132 (7.5 μg/ml) for 30-60 minutes. Nuclear proteins (5 μg/reaction) were used for EMSA.

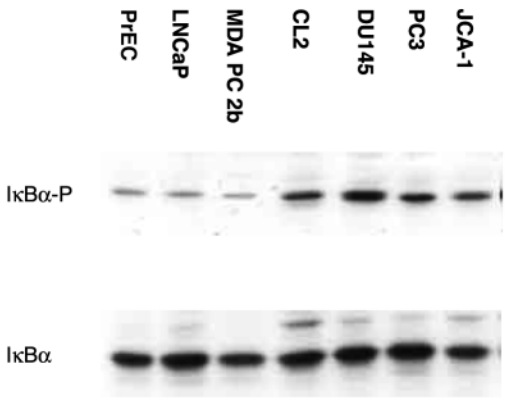

Fig. 5. Analysis of IκBα phosphorylation in androgen-independent PC cells. Western blots containing whole cell protein extracts (10 μg/lane) from untreated cells were probed for expression of IκBα and IκBα-P. Data are shown for the one representative experiment.

Results presented in Fig. 5 clearly show that IκBα is heavily phosphorylated in androgen-independent DU145, PC3, and JCA1. We would like to emphasize that, in several experiments, the highest level of IκBα-P was found in DU145 cells with the highest constitutive κB activity. We also found that level of IκB-α phosphorylation was higher in androgen-independent CL2 cells than in LNCaP cells from which they were derived (Fig. 5).

Further, we assessed the rate of constitutive IκBα phosphorylation in LNCaP cells with low and DU145 cells with high constitutive activity of NF-κB. To evaluate the rate of IκBα phosphorylation we used proteasomal inhibitor MG132 to block degradation of phosphorylated IκBα (Sun and Carpenter, 1998). MG132 treatment resulted in accumulation of phosphorylated IκBα protein in both cell lines, however the rate of IκBα-P accumulation was faster and the final amount of the phosphorylated IκBα protein was much higher in DU145 cells compared with LNCaP cells (Fig. 6).

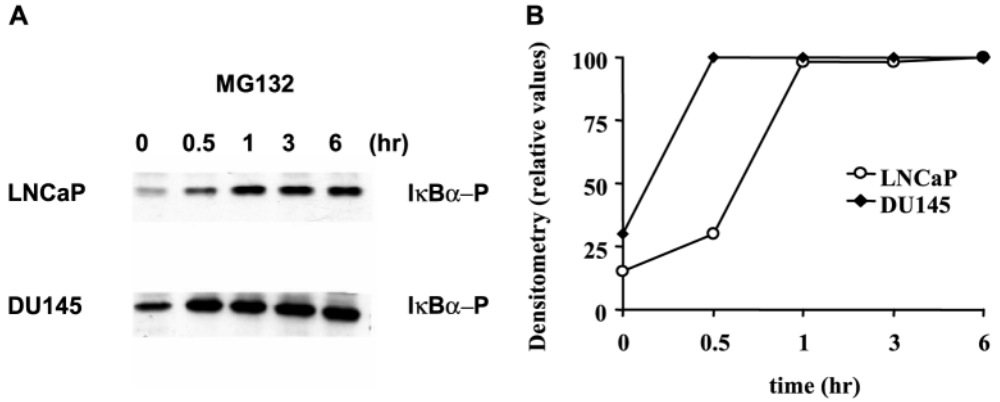

Fig. 6. The rate of IκBα phosphorylation in androgen-dependent and androgen-independent PC cell lines. Indicated cell lines were treated with proteasomal inhibitor MG132 (7.5 μg/ml) for 0.5-6 hours. (A) Western blots containing cytosol proteins (10 μg/lane) were probed for expression of IκBα-P. (B) Western blots shown in A are plotted as a percentage of the maximum IκBα-P expression level. Abscissa: time after MG132 treatment (hours). Ordinate: relative amount of IκBα-P. Data are shown for the one representative experiment.

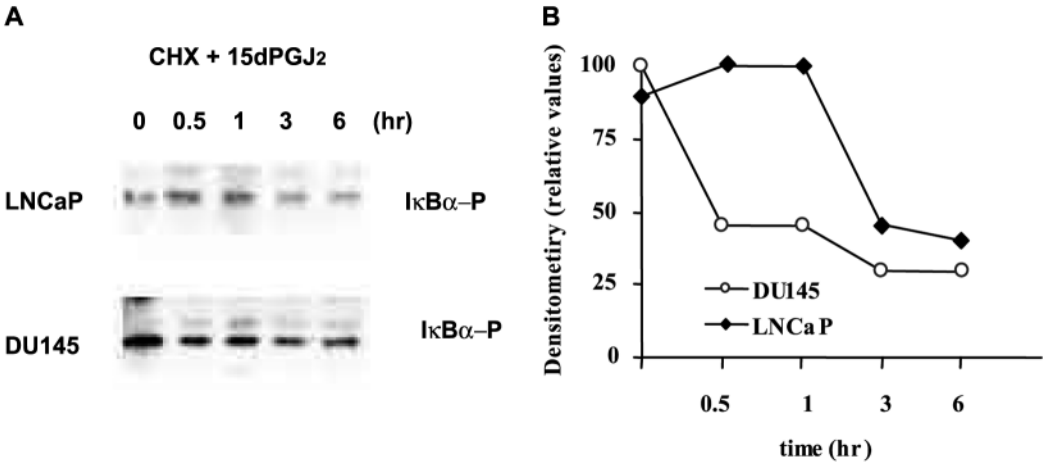

Fig. 7. The rate of IκBα-P degradation is higher in androgen-independent PC cells. Indicated cell lines were treated with cycloheximide (CHX, 10 μg/ml) in combination with 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15dPGJ2) for 0.5-6 hours. (A) Western blots containing cytosol protein extracts (10 μg/lane) were probed for expression of IκBα-P. (B) Western blots shown in A are plotted as a percentage of the initial IκBα-P expression level. Abscissa: time after treatment (hours). Ordinate: relative amount of IκBα-P. Data are shown for the one representative experiment.

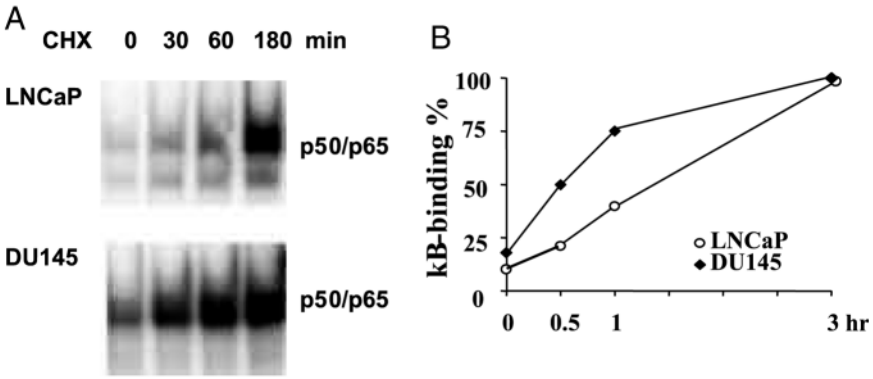

Fig. 8. Effect of cycloheximide on NF-κB binding in PC cells. Indicated cell lines were treated with CHX (10 μg/ml) for 0.5-3 hours. Nuclear proteins (5 μg/reaction) from cells were used for electrophoretic mobility shift assay (EMSA). (A) NF-κB binding activity. (B) NF-κB binding activity shown in A is plotted as a percentage of the maximum (at 3 hours of CHX treatment) p65/p50 binding level. Abscissa: time after treatment (minutes). Ordinate: relative amount of NF-κB binding. Data are shown for the one representative experiment.

Next we evaluated the rate of IκBα-P degradation in the same PC cell lines using cycloheximide (CHX), an inhibitor of protein synthesis, combined with cyclopentenone prostaglandin J2 (15dPGJ2), an inhibitor of IKK (Rossi et al., 2000). Under these conditions, de novo synthesis of IκBα as well as phosphorylation of pre-existing IκBα were blocked. Western blot analysis of pre-existing IκBα-P degradation demonstrated that the rate of degradation of IκBα-P was significantly higher in DU145 cells than in LNCaP cells (Fig. 7).

Facilitation of degradation of IκBα due to the blockage of its de novo synthesis, was expected to result in the translocation of NF-κB into the nucleus. Indeed, CHX treatment increased κB binding both in DU145 and LNCaP cells. The comparison of time curves for NF-κB activation by CHX confirmed that IκB degradation occurs at a considerably higher rate in DU145 cells than in LNCaP cells (Fig. 8).

We also directly assessed the time of IκBα half-life in those two PC cell lines using pulse-chase analysis of metabolically labeled IκBα (Fig. 9). We found that IκBα was more than twice as stable in LNCaP cells (IκBα half-life was about 60 minutes) as in DU145 cells. Conclusively, the comparative analysis of IκBα-P phosphorylation and degradation indicates that IκBα turnover is significantly greater in androgen-independent PC cells; this suggests that IKK activity should be higher in those cells.

Instability of IκBα correlates with constitutive IKK activity in PC androgen-independent cell lines

Recently several IκB kinases (IKK) that phosphorylate IκB proteins in response to diverse NF-κB activators have been identified. The IKKα and IKKβ are the major inducible IKKs (Maniatis, 1997). Western blot analysis of whole cell protein extracts from primary prostate cells and five PC cell lines with antibodies against IKKα/IKKβ did not show significant alterations in the expression of those proteins (Fig. 10A). To determine the activity of endogenous IKKs in prostate cells we performed an in vitro kinase assay. As a positive control we used a protein extract from LNCaP cells stimulated by TNF-α. The data presented in Fig. 10B demonstrate that as predicted, the constitutive IKK activity was higher in the three androgen-independent cell lines, than in primary prostate cells and androgen-dependent cell lines. Thus, constitutive IKK activation appears to be responsible for high rate of IκBα phosphorylation and ultimately for NF-κB activation in androgen-independent PC cells.

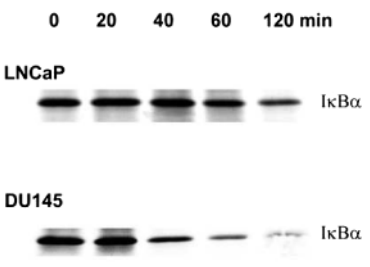

Fig. 9. Pulse-chase analysis of IκBα degradation in PC cell lines. LNCaP and DU145 cells were metabolically labeled with ^{35}S-Met-Cys and harvested at indicated time points. IκBα was immunoprecipitated, resolved on 12.5% denaturating PAAG and transferred to membrane. Dried membrane was subjected to radiography.

Effect of IKK d.n. constructs on the basal level of NF-κB activity in prostate cells

To further explore the role of IKKs in constitutive NF-κB

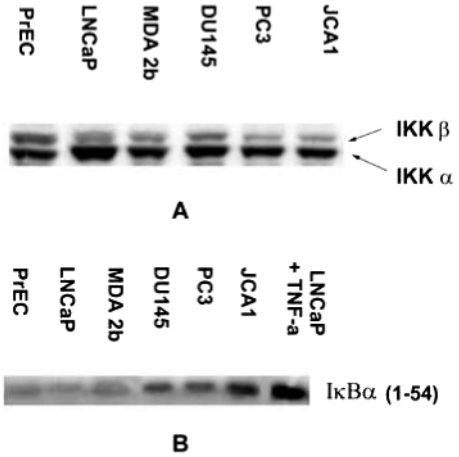

Fig. 10. Analysis of IKK expression and IKK activity in normal prostate cells and PC cell lines. (A) Western blot analysis of IKKα and IKKβ expression. Western blots containing whole cell protein extracts from untreated cells (10 μg/lane) were probed for expression of IKKα and IKKβ. (B) Analysis of IKK activity. Protein extracts from untreated cells were immunoprecipitated with a combination of IKKα and IKKβ antisera, and used for in vitro kinase reaction. Protein extract from LNCaP cells stimulated by TNF-α (7.5 ng/ml, 10 minutes) was used as a positive control.

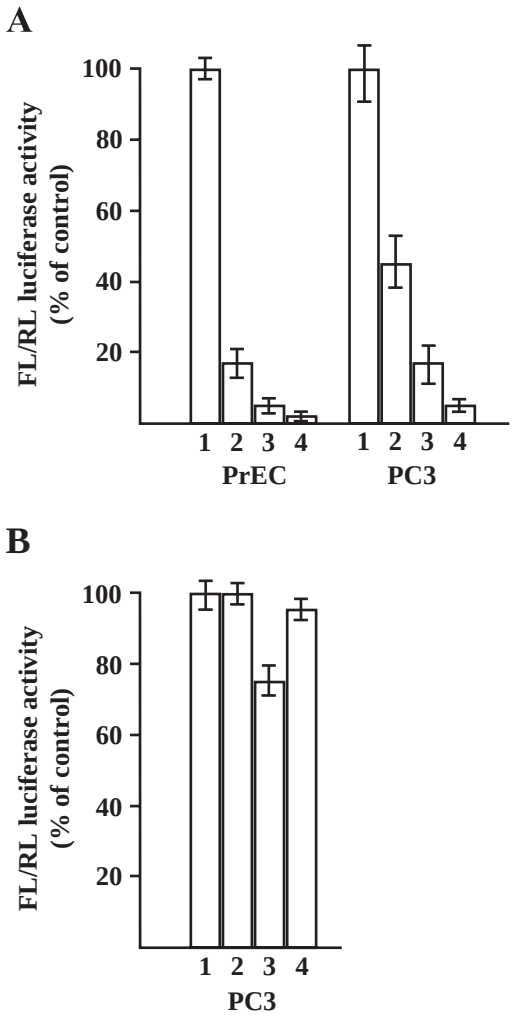

Fig. 11. Effect of d.n. IKK mutants on NF-κB constitutive activity in normal prostate cells and the PC3 cell line. (A) Prostate cells were co-transfected with κB-luciferase, pRL-null and (1) control vector; (2) IKKα d.n. mutant; (3) IKKβ d.n. mutant; and (4) IκBα d.n. mutant. (B) Prostate cells were co-transfected with MMTV-luciferase, pRL-null and (1) control vector; (2) IKKα d.n. mutant; (3) IKKβ d.n. mutant; and (4) IκBα d.n. mutant. Luciferase activity was measured 24 hours after transfection in untreated prostate cells by dual luciferase assay. Data are shown as FL/RL luciferase activities ratio (% to control) for one representative experiment. PrEC, normal epithelial prostate primary cultures.

activation in malignant prostate cells we studied the effect of kinase-inactive mutants of either IKKα (IKKαK44M) or IKKβ (IKKβK44M) on the constitutive NF-κB transcription activity in normal and malignant PC cells in comparison with the effect of IκBα d.n. mutant. Those IKK mutants are not able to phosphorylate IκBs and were shown to block IKK activity in a dominant-negative fashion in such cells as HeLa and 293 human embryonic kidney cells (O'Mahony et al., 2000). As shown in Fig. 11A, both mutants inhibited constitutive luciferase activity in normal and malignant PC3 prostate cells in a similar way, with IKKβ mutant being a more potent inhibitor for constitutively active NF-κB. The effect of the IKKβ mutant was comparable with the effect of IκBα d.n. mutant. The inhibitory effect of IKK mutants on the κB.Luc reporter was specific: IKKβ and IKKα mutants did not affect significantly the constitutive activity of MMTV.Luc in PC3 cells (Fig. 11B).

Effect of IκBα d.n. construct on basal and induced apoptosis in prostate cells

We have extended our study further and studied the biological consequences of NF-κB blockage in PC cells with the high and low constitutive NF-κB activity. We have chosen for these experiments IκBα mutant, which was able to block significantly (up to 90-95%) NF-κB activity in the luciferase reporter assay (Fig. 11). Apoptosis was determined morphologically and by poly(ADP-ribose) (PARP) cleavage. Caspase-mediated cleavage of PARP inactivates this enzyme and inhibits its ability to respond to DNA strand breaks for repair. PARP cleavage is now recognized as one of the most sensitive markers of caspase-mediated apoptosis. We found that NF-κB blockage in LNCaP cells by the IκBα d.n. mutant resulted in massive apoptosis comparable with the apoptosis induced by TNF-α. We observed profound cell retraction, rounding and detachment 24-48 hours after infection. PARP cleavage was similarly increased in LNCaP cells infected with AdV-d.n. IκBα and in LNCaP cells treated with TNF-α (Fig. 12A, lanes 2,5). Moreover, those treatments resulted in reduced expression of full-length PARP, and consequently the ratio of cleaved PARP/total PARP was dramatically affected in cells with blocked NF-κB, and especially in cells with blocked NF-κB treated with TNF-α. It is interesting that NF-κB blockage in PC3 cells resulted in significant apoptosis only when it was combined with TNF-α treatment (Fig. 12A,B). Indeed, the ratio of cleaved PARP/total PARP was high only in PC3 cells with blocked NF-κB treated with TNF-α (Fig. 12A, lane 3). Consistently, 30-40% of TNF-α-treated PC3 cells with blocked NF-κB cells demonstrated characteristic blebbing (Fig. 12B, 3). Neither infection with AdV-d.n. IκBα alone nor treatment with TNF-α alone induced changes in morphology of PC3 cells (Fig. 12B, 1,2).

Discussion

This is the first study to develop a comprehensive and detailed picture of changes in basal NF-κB activity in a panel of prostate cells including primary prostate epithelial cells, two androgen-dependent and four androgen-independent PC cell lines. We found that NF-κB was constitutively activated in human androgen-independent PC cell lines DU145, PC3, JCA1 as well as androgen-independent CL2 cells derived from LNCaP androgen-dependent cells. Thus, we confirmed the recent finding of a high NF-κB activity in some PC cell lines (Palayoor et al., 1999). Our results are also in agreement with recent findings on persistent activity of NF-κB in several other human tumors and tumor cell lines (Rayet and Gelinas, 1999; Baldwin, 1996; Wang et al., 1999; Bours et al., 1994; Nakshatri et al., 1997; Sovak et al., 1997; Visconti et al., 1997; Dejardin et al., 1995).

It is important to mention that amplification, overexpression and rearrangements of most genes coding for Rel/NF-κB factors have been found in hematopoietic tumors and could underlie the constitutive NF-κB activation (Rayet and Gelinas, 1999). However, the most frequent finding in solid tumors and

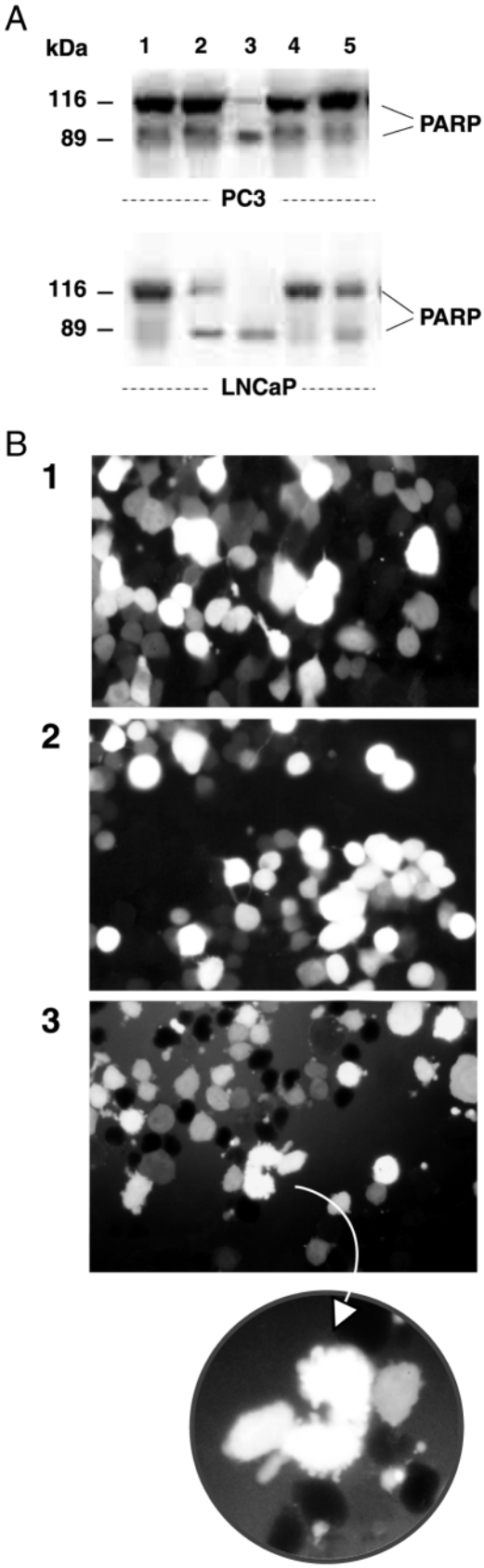

Fig. 12. Effect of IκBα d.n. mutant on apoptosis in prostate cells. (A) Western blot detection of PARP cleavage. PC3 and LNCaP prostate cells were infected with adenovirus expressing GFP and IκBα mutant lacking Ser32 and Ser36 (AdV-d.n.IκBα) or adenovirus expressing only GFP (AdV-control). 24 hours later cell cultures were left untreated or treated with TNF-α (7.5 ng) for 10 hours. PARP cleavage was detected by western blotting with antibody that detects the full length PARP (116 kDa) and PARP cleavage product (85 kDa). Adherent cells and detached floaters were combined for whole-cell lysate preparations. (1) Untreated cells; (2) AdV-d.n.IκBα-infected cells; (3) AdV-d.n.IκBα-infected cells treated with TNF-α; (4) AdV-control-infected cells; (5) AdV-control-infected cells treated with TNF-α. (B) Effect of IκBα d.n mutant on morphology of PC3 cells. Micrographs (×300) depicting representative morphological response of PC3 cells 48 hours after infection: (1) with AdV-control; (2) with AdV-d.n.IκBα; and (3) with AdV-d.n.IκBα and treated with TNF-α. Note numerous blebbing cells in cell cultures treated with TNF-α.

cell lines derived from solid tumors was the overexpression of p50 and p52 proteins (Rayet and Gelinas, 1999; Dejardin et al., 1995). p50 and p52 proteins have low transactivation activity, thus the biological role of p50 and p52 homodimers appears to be ambiguous (Budunova et al., 1999). The participation of RelA in solid tumors is the subject of many debates. RelA exhibits strong transactivation potential, however, alteration of RelA expression/function in solid tumors or cell lines derived from solid tumors has been only rarely reported (Rayet and Gelinas, 1999). Significantly, we found that the activation of p65/RelA-containing NF-κB complexes with the highest transactivation potential among other NF-κB dimers, was specific for PC cell lines and occurred without p65 or p50 overexpression in androgen-independent PC cells. In this regard it is important that nuclear p65 expression was increased in prostate carcinomas compared to surrounding apparently normal tissues.

The altered expression of IκBs as well as mutations in IκB genes in tumor cells are implicated in the constitutive activation of NF-κB (Rayet and Gelinas, 1999; Emmerich et al., 1999; Cabannes et al., 1999). However, the results of our experiments strongly suggest that constitutive activation of NF-κB in PC cells is not a consequence of either altered expression or large rearrangements or mutations in NF-κB/IκB genes. Indeed, we did not find any changes in the level of expression of p65, p50 and three major IκB proteins (IκBα, IκBβ and IκBε) as well as deviations from expected sizes of those molecules in PC cells with activated NF-κB. Further, direct sequencing of IκBα cDNA has not predicted any mutations of the IκBα protein in cell lines with constitutive NF-κB activation. We cannot presently rule out the presence of mutations in IκBβ, IκBε, p50 or RelA genes in DU145, PC3 and JCA1 cells. However, our experiments with different NF-κB inhibitors and activators provided indirect evidence that NF-κB is normally controlled by IκBs and fully functional in those PC cells. Indeed, the constitutive activity of NF-κB in DU145, PC-3 and JCA1 cells was inhibited by the IKKα d.n. mutant, IKKβ d.n. mutant and by a proteasomal inhibitor MG132 that effectively blocks degradation of all IκB proteins (Sun and Carpenter, 1998). The analysis of the sensitivity of PC cells to the standard NF-κB inducers such as TNF-α, LPS and TPA, revealed that, in contrast to the Hodgkin lymphoma cells (Krappmann et al., 1999), and in spite of the high basal level of NF-κB activity, PC cells are highly sensitive to NF-κB activation (Gasparian et al., 2000).

Another recently described mechanism of NF-κB activation in tumor cells implicates increased IκBα phosphorylation and turnover (Devalaraja et al., 1999; Krappmann et al., 1999). We found that in all studied androgen-independent PC cells, including CL2 cells derived from LNCaP cells, IκBα was heavily phosphorylated. Moreover, IκBα displayed a faster turnover in androgen-independent PC cells than in androgen-dependent PC cells. In addition, by in vitro kinase assay we demonstrated constitutive activation of IKK in androgen-independent cell lines. It is currently understood that the mechanisms of basal and induced NF-κB activation could be different. Activation of NF-κB through phosphorylation, ubiquitination and proteasome-dependent degradation of IκBs is specific for cells treated with NF-κB inducers (Whiteside and Israel, 1997; Heissmeyer et al., 1999). The mechanisms responsible for the maintenance of the basal NF-κB activity are less clear and may not require IκBα phosphorylation at Ser32/36, ubiquitination or even proteasome-dependent degradation (Miyamoto et al., 1998; Krappmann et al., 1996). Our data strongly suggest that in androgen-independent PC cells, basal NF-κB activation employs a mechanism similar to that for NF-κB activation by inducers such as cytokines. It appears that constitutive NF-κB activity depends on the constitutive aberrant activation of IKKs and consequently, a faster IκBα turnover.

In this regard, it is important to mention that the androgen-independent PC cells produce numerous growth factors and cytokines, that are strong activators of IKK complex and consequently NF-κB. Those cytokines and growth factors include TNF-α, different interleukins, FGF, EGF, NGF, HGF, PDGF and VEGF (Baldwin, 1996; Sun and Carpenter, 1998; Byrd et al., 1999; Gentry et al., 2000; Romashkova and Makarov, 1999). Knowing that the expression of genes encoding certain cytokines, for example *IL-6*, is regulated by NF-κB (Zhang et al., 1994), one could assume that activation of IKK in PC cells involves an established positive autocrine/paracrine loop.

Androgen-independent cell lines used in this study do not express androgen receptor (AR) (Tso et al., 2000; Mitchell et al., 2000). This allows to find an interesting parallel between NF-κB activation in androgen-independent PC cells and estrogen receptor (ER)-deficient breast carcinoma cell lines (Nakshatri et al., 1997; Biswas et al., 2000) and to raise the question of the possible role of NF-κB in the development of growth autonomy and resistance to apoptosis in hormone-independent prostate and breast tumors. It is known that NF-κB is a key anti-apoptotic factor in most cells (Barkett and Gilmore, 1999). It has become clear recently that NF-κB could also play the pro-proliferative role in some cells through direct activation of genes involved in the cell cycle (Biswas et al., 2000; Hinz et al., 1999; Guttridge et al., 1999).

We found that NF-κB blockage resulted in the increased apoptosis in LNCaP cells, and increased sensitivity to apoptosis induced by TNF-α in PC3 cells with high constitutive NF-κB activity. The latter result is in line with the previous finding on the essential role of NF-κB in resistance of PC cells to TNF-α (Sumitomo et al., 1999). The high resistance of PC3 cells with elevated constitutive level of NF-κB, to NF-κB blockage could be explained by the residual NF-κB activity in those cells (data not shown).

It is important to mention that despite some general similarities in the response of prostate cells to androgens and NF-κB inducers, there is an evidence that NF-κB and AR mutually repress each other transcriptional activity. The repression involves either direct protein-protein interaction between AR and p65 or competition for intracellular transcriptional regulators (Palvimo et al., 1996; Valentine et al., 2000). Moreover, crosstalk between signaling mediated by AR and NF-κB also involves transcriptional repression of the *AR* gene by NF-κB (Supakar et al., 1995). This suggests that NF-κB blockage may result in restoration of AR function in PC cells.

In conclusion, the results presented here demonstrate that aberrant IKK activation leads to the constitutive activation of the NF-κB 'survival signaling' pathway in androgen-independent PC cells. Since NF-κB protects prostate cells from apoptosis, possibly stimulates proliferation of PC cells, and plays an important role in the selection for hormone-independence, NF-κB and IKK inhibition may prove useful both in the prevention of PC and in adjuvant therapy. Further studies are needed to identify the affected upstream signaling that results in IKK activation.

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Selenium Compounds Inhibit IκB Kinase (IKK) and Nuclear Factor-κB (NF-κB) in Prostate Cancer Cells[1]

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Abstract

Selenium compounds are potential chemopreventive agents for prostate cancer. There are several proposed mechanisms for their anticancer effect, including enhanced apoptosis of transformed cells. Because the transcription factor nuclear factor-κB (NF-κB) is often constitutively activated in tumors and is a key antiapoptotic factor in mammalian cells, we tested whether selenium inhibited NF-κB activity in prostate cancer cells. In our work, we used sodium selenite and a novel synthetic compound, methylseleninic acid (MSeA), that served as a precursor of the putative active monomethyl metabolite methylselenol. We found that both selenium forms inhibited cell growth and induced apoptosis in DU145 and JCA1 prostate carcinoma cells. Sodium selenite and MeSeA, at the concentrations that induced apoptosis, inhibited NF-κB DNA binding induced by tumor necrosis factor-α and lipopolysaccharide in DU145 and JCA1 prostate cells. Both compounds also inhibited κB.Luciferase reporter activity in prostate cells. A key to NF-κB regulation is the inhibitory κB (IκB) proteins that in response to diverse stimuli are rapidly phosphorylated by IκB kinase complex, ubiquitinated, and undergo degradation, releasing NF-κB factor. We showed that sodium selenite and MSeA inhibited IκB kinase activation and IκB-α phosphorylation and degradation induced by TNF-α and lipopolysaccharide in prostate cells. NF-κB blockage by IκB-α d.n. mutant resulted in the sensitization of prostate carcinoma cells to apoptosis induced by selenium compounds. These results suggest that selenium may target the NF-κB activation pathway to exert, at least in part, its cancer chemopreventive effect in prostate.

Introduction

Prostate cancer is the second leading cause of cancer death in men in the United States. Despite significant improvement of hormonal, chemical, and radiation therapies, there is no cure for locally advanced or metastatic prostate cancer. Thus, studies to develop strategies for prostate cancer prevention continue to be essential. During the past two decades, selenium emerged as a factor with the most consistent anticancer effect among a number of micronutrients tested in animal experiments and clinical trials (1–7). Significantly, selenium appeared to be one of the most promising agents for prostate cancer prevention. The most convincing data have been obtained by Clark *et al.* (3) in a double-blind, placebo-controlled trial involving >1000 patients. Participants treated with a supranutritional dose of selenium in the form of selenized brewer's yeast for 4.5 years (200 μg of selenium daily, with the average daily intake of selenium in the United States ~100 μg) had substantial reductions in the incidence of prostate cancer, as well as total cancer incidence and mortality (3, 4). The follow-up for the cohort used in Clark's trial further indicated that selenium treatment decreased prostate cancer risk (8).

Selenium is an essential trace element nutrient and is a normal component of diets. The nutritional essentiality of selenium is linked to the functional activities of several enzymes and proteins that contain selenium, including glutathione peroxidases, thioredoxin reductases, and others (2, 9, 10). The chemopreventive activity of selenium is determined by its various covalent forms and metabolites. Over the past 15 years, several groups have focused on the identification of the active metabolite(s) that is critical in selenium cancer chemoprevention and on the search of less toxic forms of selenium that would retain its chemopreventive activity. Selenite is metabolized *in vivo* to a key proximal metabolite hydrogen selenide, which in turn undergoes sequential methylation to methylselenol and other methyl-selenium metabolites (1). Because methylation of hydrogen selenide produces forms of selenium that are not genotoxic (11), stable methylated selenium compounds are currently viewed as precursors or "pro-drugs" to release methylselenol and hold a high potential for cancer chemoprevention use. MSeA[4] is one of such synthetic compounds that effectively inhibited tumor cell growth *in vitro* (12) and possessed strong antitumor activity in animal experiments (13).

Although several mechanisms including antioxidant protection (via glutathione peroxidases), altered carcinogen metabolism, enhanced immune surveillance, and inhibition of neoangiogenesis have been proposed to account for the anticancer effect of selenium (9, 11), induction of apoptosis

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[4] The abbreviations used are: MSeA, methylseleninic acid; IκB, inhibitor of NF-κB; IKK, IκB kinase; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; PARP, poly(ADP-ribose) polymerase; GFP, green fluorescent protein; TNF, tumor necrosis factor; EMSA, electrophoretic mobility shift assay; EMSSA, electrophoretic mobility supershift assay; FL, *Fireflight* luciferase; RL, *Renilla* luciferase.

of tumor cells by selenium may be of special significance in the chemoprevention of prostate cancer, which is known for very low proliferative activity (14). Because the transcription factor NF-κB is a key antiapoptotic factor in mammalian cells, we hypothesized that suppression of the NF-κB activation pathway may be related to selenium chemopreventive activity in prostate.

The active NF-κB complex is a homo- or heterodimer composed of proteins from the NF-κB/Rel family. In nonstimulated cells, NF-κB resides in the cytoplasm in a complex with the inhibitor protein, collectively called IκB (15, 16). Most agents that activate NF-κB use a common pathway based on the phosphorylation of the two NH_2-terminal serines in IκB molecules IκB-α, IκB-β, and IκB-ε with subsequent ubiquitination and degradation of IκB proteins by the 26S proteasome (17–21). Degradation of IκBs results in nuclear translocation of released NF-κB dimers (p65/p50) and activation of target genes. Signal-induced phosphorylation of IκB is executed by the recently identified IKK complex containing IKKα and IKKβ and regulatory proteins (22).

Although selenium has a strong potential as chemopreventive agent for prostate, the mechanisms underlying its diverse biological effects have been studied mostly in other cancer cell models but not in prostate cells. To test our hypothesis that suppression of the NF-κB activation pathway may be involved in selenium chemopreventive activity in prostate, we studied the effect of two selenium compounds, selenite and MSeA, on NF-κB activity and upstream IKK kinases in prostate carcinoma cell lines in parallel with the effect of those compounds on prostate cell growth and apoptosis.

Materials and Methods

Cell Cultures and Treatments. In our work, we used two androgen-resistant prostate carcinoma cell lines, DU145 and JCA1. DU145 prostate cancer cells were purchased from American Type Culture Collection (Rockville, MD). JCA1 prostate cancer cells, originally established by Muraki *et al.* (23), were obtained from Dr. O. Rokhlin (University of Iowa, Iowa City, IA). Both cell lines were cultured in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 10% FBS (HyClone, Logan, UT), 10 mM HEPES, 1 mM sodium pyruvate, 0.01 mM 2-mercaptoethanol, 2 mM L-glutamine, and antibiotics as described elsewhere (24). Cells were treated with LPS (from *Escherichia coli* 026:B6; Sigma Chemical Co, St. Louis, MO), TNF-α (R&D Systems, Minneapolis, MN), sodium selenite pentahydrate (J. T. Baker, Inc., Phillipsburg, NJ), or MSeA (a generous gift from Dr. H. Ganther, University of Wisconsin at Madison, Madison, WI).

Effect of Selenium Compounds on Cell Accumulation and Apoptosis in Prostate Cell Cultures. To study the effect of selenium on cell accumulation, DU145 and JCA1 cells were plated on 35-mm dishes and cultured to 50% confluence. They were treated with MSeA or selenite at a concentration of 1–5 μM without medium change for 24 h. The number of cells in three dishes/treatment was determined.

We used PARP proteolysis and DNA fragmentation assays to determine apoptosis 24 h after the beginning of selenium treatment. Adherent cells and detached floating cells were combined for whole-cell protein extract preparations. PARP cleavage was estimated by Western blot analysis with PARP antibodies (PharMingen, San Diego, CA).

For DNA fragmentation assay, DNA was isolated from adherent cells and detached floating cells as described previously (12). Briefly, cells were lysed in a buffer containing 10 mM Tris-HCl (pH 8.0), 100 mM EDTA, 0.5% SDS, 0.5 mg/ml proteinase K, and digested at 50°C for 3 h. The lysate was extracted twice with phenol-chloroform. Nucleic acids were precipitated with isopropanol in the presence of 0.2 M NaCl. The pellet was resuspended in 30 μl of 10 mM Tris-HCl, 1 mM EDTA (pH 7.5), treated with RNase, and loaded onto 1.5% agarose gel containing 0.1 μg/ml ethidium bromide for electrophoresis. Gels were photographed using UV illumination.

NF-κB Blockage by Adenovirus Infection. Prostate cells were seeded on 35-mm dishes and at 50% confluence were infected with type 5 recombinant adenovirus (AdV) construct AdV-d.n.IκBα encoding GFP and mutant human IkBα protein with substitution of serines 32 and 36 to alanines (32A36A) or adenovirus encoding only GFP (AdV-control). AdV-d.n.IkBα virus with deletions of E1 and E3 was generated using the AdEasy1 system. The AdEasy1 system was a generous gift of Dr. T-C. He, (The Howard Hughes Medical Institute, Baltimore, MD). Mutations of IkBα were constructed by site-directed mutagenesis with the Bio-Rad Muta-Gene Phagemid In Vitro Mutagenesis system (Bio-Rad Laboratories, Hercules, CA). IkBα mutant has an NH_2-terminal tag (ADRRIPGTAEENLQK) derived from the equine infectious anemia virus tat protein. Control E1/E3-deleted AdV 5 with GFP (AdV-control) was purchased from Quantum Biotechnologies (Montreal, Canada). Adenoviruses were purified by CsCl gradient centrifugation. Cells were infected with adenoviruses (10^9 viral particles/dish) in 700 μl of medium with 0.5% serum overnight. Twenty-four h after infection, cells were treated with selenium compounds for 24 h.

Preparation of Nuclear and Cytosol Protein Extracts. Cells ($20–25 \times 10^6$) cells were used for each time point. Cells were washed and harvested in cold PBS. Cell pellets were resuspended in homogenization buffer, and nuclear and cytosolic protein extracts were prepared as described previously (25).

EMSA. The binding reaction for EMSA contained 10 mM HEPES (pH 7.5), 80 mM KCl, 1 mM EDTA, 1 mM EGTA, 6% glycerol, 0.5 μg of poly(deoxyinosinic-deoxycytidylic acid), 0.5 μg of sonicated salmon sperm DNA, γ-^{32}P-labeled ($2–3 \times 10^5$ cpm) double-stranded κB-consensus oligonucleotide (Promega Corp., Madison, WI), and 5–10 μg of the nuclear extract proteins. DNA-binding reaction was performed at room temperature for 30–45 min in a final volume of 20 μl. To determine the composition of NF-κB complexes, 1.5 μl of antibodies against p65 (sc-109X) or p50 (sc-114X) were added 30 min after the beginning of reaction, and incubation was continued for additional 30–45 min. Both antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). DNA-protein complexes were analyzed on 6% DNA retardation gels (Novex, Carlsbad, CA). Dried gels were subjected to autoradiography.

Transient Transfection and Measurement of Luciferase Activity. JCA1 prostate cells were plated on 35-mm dishes and at 50% confluency were cotransfected by Tfx-50 reagent (Promega Corp.) with 3× κB.Luciferase reporter-FL under promoter with three copies of conventional κB site (Clontech Laboratories, Inc., Palo Alto, CA) and pRL-null construct-RL under minimal promoter (Promega Corp.). Tfx-50 reagent (2.25 μl/μg of plasmid DNA) and the plasmid DNAs (both in doses of 2 μg/dish) were added to the dishes in antibiotic-free, serum-free medium. Twenty-four h after transfection, JCA1 cells were pretreated with selenium compounds for 30 min and treated with TNF-α for additional 6 h. Cells were harvested in the lysis buffer, and the luciferase activity was measured by dual luciferase assay (Promega Corp.) as recommended by the manufacturer. FL activity was normalized against RL activity to equalize for transfection efficacy.

Western Blot Analysis. Nuclear or cytosol proteins were resolved by electrophoresis on 10–12.5% SDS-PAGE, depending on the size of the target proteins, and transferred to Immobilon-P membrane (Millipore Corp., Bedford, MA). Polyclonal anti-p65 (sc-372) and anti-IκBα (sc-371) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Ser32 IκBα antibody was from Cell Signaling Technology, Inc. (Beverly, MA). Monoclonal PARP antibody that recognizes both PARP and cleaved PARP was purchased from PharMingen. Membranes were blocked with 5% nonfat milk in TBST buffer [10 mM Tris HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20] and incubated with primary antibodies for 1.5 h at room temperature. Anti-phospho-Ser32 IκBα antibody and PARP antibody required 6 h incubation at 34°C. Peroxidase-conjugated antirabbit or antimouse IgG (Sigma Chemical Co.) was used as a secondary antibody. ECL Western blotting detection kit (Amersham Pharmacia Biotech, Uppsala, Sweden) was used for protein detection. The membranes were also stained with Ponceau Red to verify that equal amounts of proteins were loaded and transferred. Quantitative analysis of Western blots was done by One-Dscan gel and blot analysis software, Scanalytics, Inc. (Fairfax, VA).

***In Vitro* IKK Activity Assay.** Cells were lysed in TNT buffer [20 mM Tris HCl (pH 7.5), 200 mM NaCl, 1% Triton X-100] with protease inhibitors (25). Immunoprecipitation of 450 μg of total protein was performed with 1 μl of rabbit IKKα (#1997) and IKKβ (#4137) antisera (a kind gift of Dr. N. R. Rice, National Cancer Institute, Frederick, MD) in 3 ml of TNT buffer. Two h later, 20 μl of protein A-Sepharose 4B (Sigma Chemical Co.) in TNT buffer were added to each sample and incubated with gentle rotation overnight. Immunoprecipitate was washed three times in TNT buffer with protease inhibitors and two times with kinase buffer without protease inhibitors. Kinase reaction was performed in kinase buffer [20 mM HEPES (pH 7.4), 2 mM $MgCl_2$, and 2 mM $MnCl_2$], containing 2 μCi of [γ-^{32}P]ATP and IκBα peptide (1–54) that has only Ser-32 and Ser-36 sites of phosphorylation (Boston Biologicals, Inc., Boston, MA) as a substrate for 30 min at 30°C. Then 2× Tricine/SDS sample buffer (Novex, Carlsbad, CA) was added to each reaction, and samples were boiled and subjected to electrophoresis on 10–20% gradient tricine PAAG (Novex). Gels were dried and exposed to film with an intensifying screen at −70°C.

Data in all figures are shown as results of representative experiments. All experiments were repeated at least three times.

Results

Effect of Selenium Compounds on Prostate Carcinoma Cell Growth and Apoptosis. Clark *et al.* (3) found that the average plasma selenium level in participants treated with a supranutritional dose of selenium in prevention trial was around 2.5 μM. To establish an effective dose of selenium compounds for NF-κB *in vitro* studies, we first tested the effects of selenite and MSeA in concentration ranging from 1 to 5 μM on cell growth in the DU145 and JCA1 prostate cell lines. Both selenium compounds inhibited cell accumulation within 24 h of treatment in a dose-dependent manner (Fig. 1). Selenite appeared to be more effective than MSeA in both cell lines; it inhibited cell accumulation at all tested concentrations (1–5 μM). MSeA substantially inhibited cell accumulation only at 5 μM.

Apoptotic effects of selenite and MSeA at 5 μM have been studied in DU145 cells using as end points PARP cleavage and DNA fragmentation. Caspase-mediated cleavage of PARP inactivates this enzyme and inhibits its ability to respond to DNA strand breaks for repair. PARP cleavage is now recognized as one of the most sensitive markers of caspase-mediated apoptosis. As shown in Fig. 2*A,* at 24 h of exposure both compounds induced PARP cleavage. Consistent with our early findings (12), MSeA induced PARP cleavage more effectively than sodium selenite. As shown in Fig. 2*B,* at 24 h of exposure both compounds strongly and to the same extent induced nucleosomal fragmentation typical for apoptotic cell death.

Selenium Compounds Inhibited NF-κB DNA Binding Activity. To determine whether inhibition of prostate cell growth and induction of apoptosis correlated with selenium effect on NF-κB function, we studied the effect of selenite and MSeA on NF-κB DNA binding in DU145 and JCA1 cell lines, both of which we have characterized previously with regard to the basal and inducible NF-κB activity (26). On the basis of the data presented above, we have chosen the 5 μM dose for both selenium compounds to study their effects on NF-κB activation in prostate cells.

Because of the greater growth inhibitory response of JCA1 cells to selenium treatment (Fig. 1), we examined this cell line first for the impact of 30-min selenium preexposure on the TNF-α-induced NF-κB DNA binding activity by EMSA. Consistent with our previous findings, TNF-α strongly induced NF-κB DNA-binding in JCA1 cells (Fig. 3). MSeA-exposed cells showed considerably inhibited κB-binding at 3 h of TNF-α stimulation, but this effect was abolished by 6 h (Fig. 3). On the other hand, selenite-exposed cells had nearly full κB-binding activity by 3 h of stimulation but displayed much less binding by 6 h, indicating a more delayed action than that of MSeA. Analysis of NF-κB binding complexes by EMSA revealed that the major NF-κB complex in JCA1 cells is represented by the p50/p65 heterodimer (Fig. 3*C*).

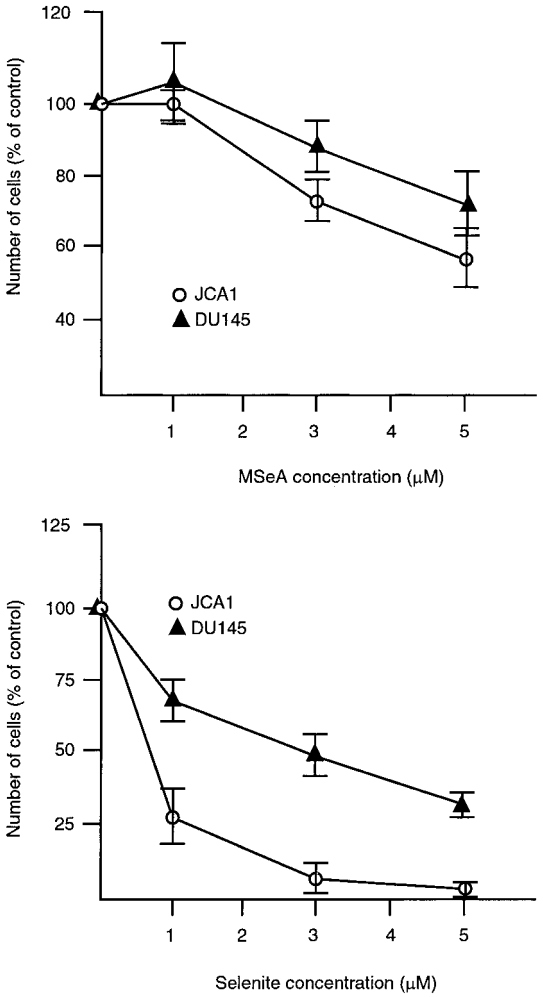

Fig. 1. Effect of selenium compounds on cell number accumulation in prostate cell cultures. DU145 and JCA1 cells were seeded in 12-well plates. At 50% confluence, cells were treated with MSeA or selenite at the indicated concentrations without medium change. The average number of cells in 3 wells/treatment was determined 24 h after the beginning of treatment. Data are shown as a percentage of control (means; *bars,* SD) for one representative experiment.

Similar patterns of differential impacts of selenite and MSeA on TNF-α-induced NF-κB DNA binding activity were observed in DU145 cells (Fig. 4*A*). The kinetics of the inhibitory action and recovery in DU145 cells appeared to be faster than in JCA1 cells. Specifically, MSeA-exposed DU145 cells showed almost a complete block of NF-κB binding at 0.5 h of TNF-α stimulation but almost fully recovered the binding activity by 3 h of stimulation. On the other hand, selenite-exposed DU145 cells showed considerably less binding by 0.5 h of TNF-α stimulation, and this effect persisted for 3 h. Selenite and MSeA preexposure also inhibited NF-κB DNA binding induced by LPS (Fig. 4*B*). The major inducible NF-κB complex in DU145 cells consisted of p50 and p65 proteins (Fig. 4*C*).

Taken together, the data support an inhibitory effect of selenium preexposure on the inducible NF-κB DNA binding activity irrespective of the nature of stimulation. Furthermore,

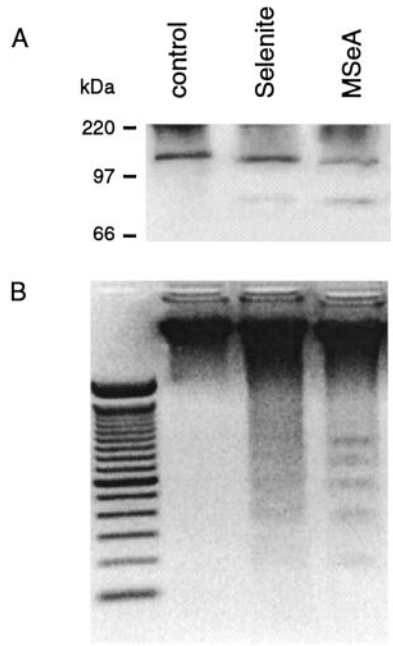

Fig. 2. Effect of selenium compounds on apoptosis in DU145 prostate cells. *A*, effect of selenium compounds on PARP cleavage. DU145 cells were treated with sodium selenite (5 μM) and MSeA (5 μM) for 24 h. PARP cleavage was defined by Western blotting with antibody, which detects both full-length PARP (116 kDA) and PARP cleavage product (85 kDa). Adherent cells and detached floaters were combined for whole-cell lysate preparations. B, effect of selenium compounds on DNA fragmentation. Agarose gel (1.5%) electrophoretic detection of nucleosomal DNA fragmentation in DU145 cells at 24 h of exposure with sodium selenite (5 μM) and MSeA (5 μM). The *leftmost lane* was loaded with DNA molecular weight marker. DNA was extracted from adherent cells combined with detached floaters. The inverted image was used to better show fragmented DNA.

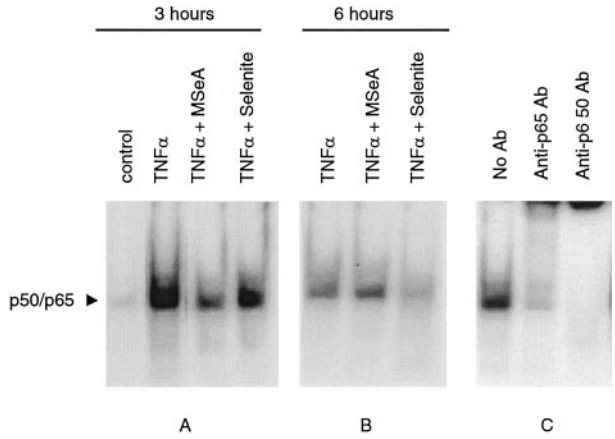

Fig. 3. Effect of selenium compounds on induced κB binding in JCA1 prostate cells. *A* and *B,* EMSA analysis of κB binding. Cells were pretreated for 30 min with MSeA (5 μM) or sodium selenite (5 μM) without medium change. Then TNF-α (3.5 ng/ml) was added to the medium, and incubation was continued. Cells were harvested at the indicated time points, and nuclear and cytosol extracts were prepared. EMSA was performed by incubating nuclear proteins with a labeled κB-oligonucleotide. *C*, identification of nuclear κB binding complexes was analyzed by EMSSA. Nuclear proteins from JCA1 cells were incubated with a labeled κB nucleotide and antibodies against p50 and p65 proteins, and DNA binding was analyzed.

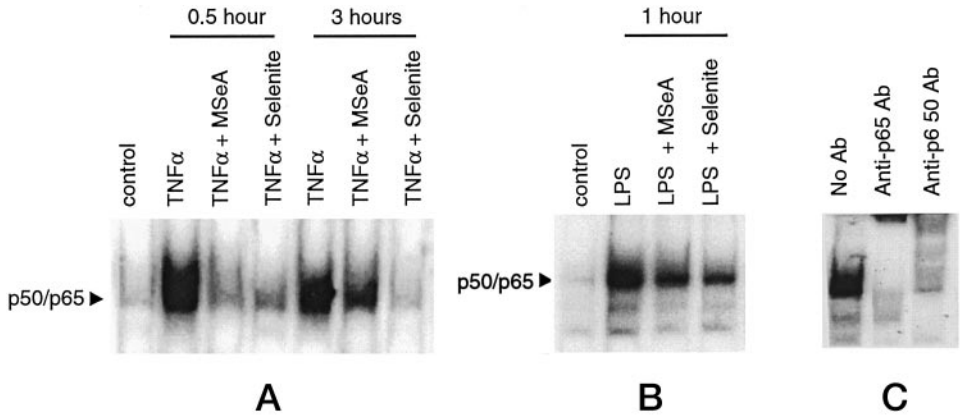

Fig. 4. Effect of selenium compounds on induced κB binding in DU145 prostate cells. *A* and *B,* EMSA analysis of κB binding. DU145 cells were pretreated for 30 min with MSeA (5 μM) or sodium selenite (5 μM). Then TNF-α (7.5 ng/ml) or LPS (1.5 μg/ml) were added to the medium, and incubation was continued. Cells were harvested at the indicated time points, and nuclear and cytosol extracts were prepared. EMSA was performed by incubating nuclear proteins with a labeled κB-oligonucleotide. *C*, identification of nuclear κB binding complexes was analyzed by EMSSA. Nuclear proteins from DU145 cells were incubated with a labeled κB nucleotide and antibodies against p50 and p65 proteins, and DNA binding was analyzed.

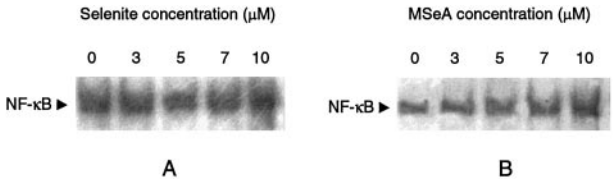

Fig. 5. Selenium compounds did not inhibit κB DNA binding in cell-free protein extracts. Nuclear proteins (10 μg/lane) from TNF-α-treated DU145 cells were incubated with a labeled κB-oligonucleotide for 45 min and used for EMSA. MSeA and sodium selenite were added to the reaction mixture at the final concentrations 1–10 μM for 15 min before the oligonucleotide.

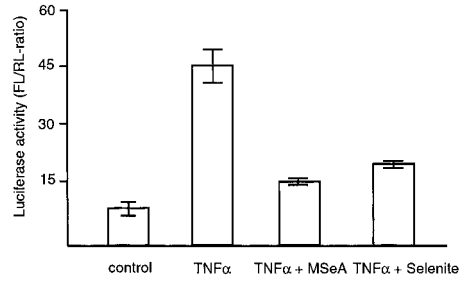

Fig. 6. Effect of selenium compounds on NF-κB transcriptional activity in prostate cells. JCA1 prostate cells were cotransfected with 3× κB.Luciferase reporter and pRL-null construct. Twenty-four h after transfection, cells were pretreated for 30 min with MSeA (5 μM) or sodium selenite (5 μM). Then TNF-α (7.5 ng/ml) was added to the medium, and incubation was continued for 6 h. Luciferase activity was measured by dual luciferase assay in cell lysates from three dishes/point. Data are shown as FL:RL ratio (means; *bars,* SD) for one representative experiment.

persistent inhibition of NF-κB activity appeared to correlate with the stronger growth-inhibitory activity of selenite than MSeA in both cell lines.

One of the previously proposed mechanisms of selenite effect on NF-κB activity is a direct interaction between selenite and NF-κB, which results in adduct formation with the thiol groups of NF-κB proteins and subsequent alteration of NF-κB properties including DNA binding (27). To test this hypothesis, we treated nuclear extracts from DU145 cells with increasing concentrations of selenite and MSeA (3–10 μM) for 1 h, the time similar to the time of *in vivo* cell treatment with selenium in our experiments with DU145 cells. As shown in Fig. 5, no direct inactivating effect of selenium on κB binding was observed by incubating nuclear extracts containing active NF-κB complexes with both selenium compounds *in vitro*.

Selenium Inhibited NF-κB-mediated Transcription Activity. To further investigate the selenium effect on NF-κB function, we performed a transient transfection of prostate carcinoma JCA1 cells with an exogenous κB-responsive gene, *κB.Luciferase* reporter. Mindful of the apoptotic effects of prolonged selenium exposure, we have chosen 6-h exposure with both selenium compounds for these experiments. The results of transient transfection experiments presented in Fig. 6 correlated well with the EMSA results. Pretreatment with both selenite and MSeA at 5 μM significantly inhibited the luciferase activity induced by TNF-α in JCA1 cells.

Selenium Inhibited TNF-α and LPS-induced Activation of IKK. As we have already mentioned, the absence of a direct effect of both selenium compounds on NF-κB DNA binding in a cell-free system (see Fig. 5) suggested that selenium affects some cellular steps in the NF-κB activation pathway. One of the first key stages to NF-κB activation is the phosphorylation of IκB inhibitory proteins by IKK kinases. Thus, in our next experiment, we studied whether selenium compounds affected IKK activation and IκB-α phosphorylation in DU145 cells.

As expected, TNF-α and LPS stimulation strongly increased IKK activity and increased the level of IκB-α phosphorylation in DU145 cells (Fig. 7). Selenite and MSeA at a concentration of 5 μM significantly inhibited IKK activation and subsequently IκB-α phosphorylation induced by TNF-α and LPS. It is important to mention that the kinetics of IKK inhibition by two different selenium compounds correlated well with kinetics of inhibition of κB DNA binding. The inhib-

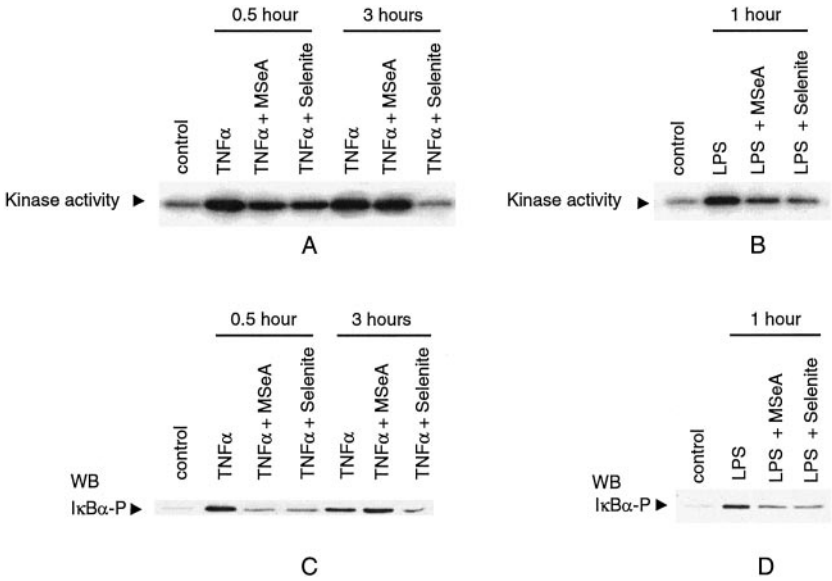

Fig. 7. Effect of selenium compounds on IKK activity and IκB-α phosphorylation in prostate cells. *A* and *B,* IKK kinase activity. DU145 cells were pretreated for 30 min with MSeA (5 μM) or sodium selenite (5 μM) for 30 min. Then TNF-α (7.5 ng/ml) or LPS (1.5 μg/ml) was added to the medium, and incubation was continued. Protein extracts from DU145 cells were prepared at the indicated time points, immunoprecipitated with a combination of IKKα and IKKβ antisera, and used for *in vitro* kinase reaction. *C* and *D,* IκB-α phosphorylation. In the same experiment, cells were harvested from the dishes at the indicated time points, and cytosol extracts were prepared. Western blots containing cytosol protein extracts (10 μg/lane) were probed for expression of IκBα-P.

itory effect of selenite on IKK activity induced by TNF-α was more sustained than the effect of MSeA.

Selenium Decreased IκB-α Degradation and p65 Nuclear Translocation. As we showed above, both selenium compounds blocked IKK activation and IκB-α phosphorylation. This suggested that proteolytic degradation of IκB-α should be also affected by selenium compounds. To determine whether selenium compounds indeed inhibited IκB-α degradation, the cytoplasmic level of IκB-α has been examined by Western blot analysis in DU145 cells pretreated with selenite and MSeA and treated with TNF-α. As we reported previously (26), TNF-α induced dramatic IκB-α degradation within 10–30 min with complete IκB-α resynthesis at later times of exposure in DU145 cells (Fig. 8). Both selenite and MSeA inhibited IκB-α degradation during the first 30 min of cell exposure to TNF-α (Fig. 8). At later times, the effect of selenium compounds on IκB-α level has not been revealed.

Whether selenium compounds affected TNF-α-induced nuclear translocation of the p65 subunit of NF-κB was examined by Western blot analysis of nuclear protein extracts. As shown in Fig. 8, upon TNF-α treatment, p65 translocated to the nucleus in DU145 cells, and both selenite and MSeA inhibited this translocation. Consistent with the results on the repression of κB DNA binding, the inhibitory effect of selenite on p65 translocation was more sustained than the effect of MSeA at 3 h.

NF-κB Blockage Sensitized Prostate Carcinoma Cells to Apoptosis Induced by Selenium Compounds. We have extended our study further and studied the effect of NF-κB blockage on the sensitivity of prostate carcinoma cells to apoptosis induced by selenium compounds. We have chosen for these experiments an IκBα mutant with substitution of serines 32 and 36 to alanines (32A36A) that, because of the lack of putative phosphorylation sites, is not phosphorylated by IKKs and as a result does not undergo degradation. We have shown previously that this IκBα mutant was able to block NF-κB activity in prostate cells by 90–95% (28).

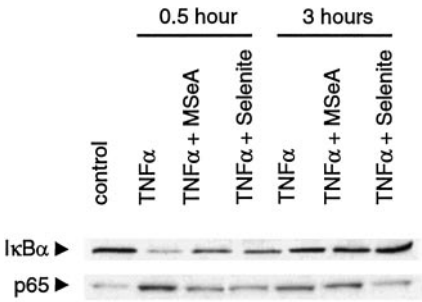

Fig. 8. Effect of selenium compounds on induced IκB-α degradation and nuclear translocation of p65 in prostate cells. Cells were pretreated for 30 min with MSeA (5 μM) or sodium selenite (5 μM). Then TNF-α (7.5 ng/ml) was added to the medium, and incubation was continued. Cells were harvested at the indicated time points, and nuclear and cytosol extracts were prepared. Western blots containing cytosol protein extracts (10 μg/lane) were probed for expression of IκBα. Western blots containing nuclear protein extracts (10 μg/lane) were probed for expression of p65.

We found that NF-κB blockage in DU145 cells by infection with adenovirus construct encoding mutant human IkBα protein (AdV-d.n.IκBα) resulted in increased sensitivity of prostate carcinoma cells to both selenium compounds. Indeed, the morphological changes in DU145 cells in response to selenium compounds, such as profound cell retraction, rounding, and detachment described in our previous work (12), occurred much earlier in cell cultures infected with adenovirus expressing IκBα mutant (data not shown).

As one could see in Fig. 9*A,* treatment of DU145 cells with sodium selenite or MSeA as well as cell infection with AdV-d.n.IκBα resulted in PARP cleavage (Fig. 9, *Lanes 3, 4,* and *5*). It is important that selenium treatment of DU145 cells with blocked NF-κB resulted in more intensive PARP cleavage than in selenium-treated cells with intact, constitutively active NF-κB (Fig. 9, compare *Lanes 3* and *6* and *Lanes 4* and *7*). Moreover, because of a reduced expression of full-length PARP in MSeA- and selenite-treated cells with blocked NF-

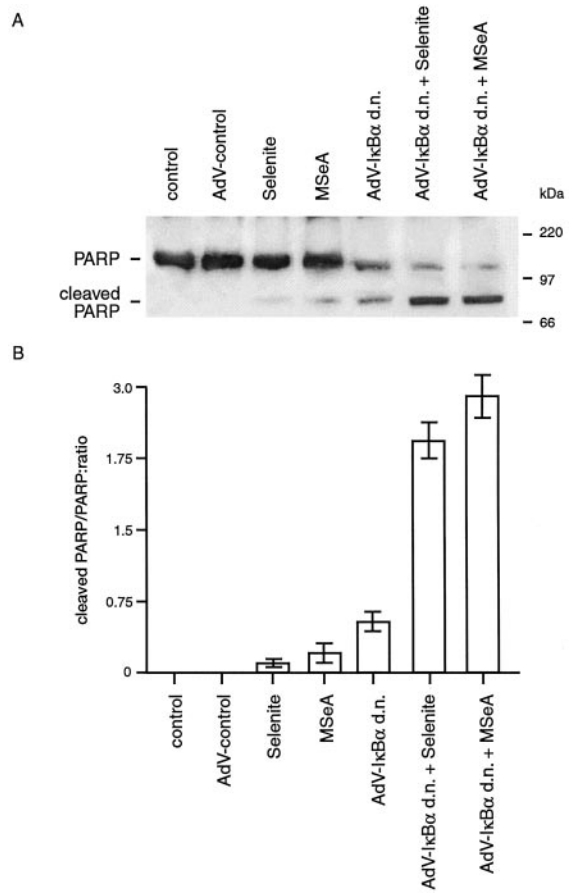

Fig. 9. Effect of IκBα d.n. mutant on PARP cleavage in prostate cells. DU145 prostate cells were infected with adenovirus expressing GFP and IκBα mutant lacking Ser-32 and Ser-36 (*AdV-d.n.IκBα*) or adenovirus expressing only GFP (AdV-control). Twenty-four h later, cell cultures were left untreated or treated with MSeA (5 μM) or with sodium selenite (5 μM) for 24 h. PARP cleavage was detected by Western blotting with antibody, which detects the full-length PARP (116 kDa) and PARP cleavage product (85 kDa). Adherent cells and detached floaters were combined for whole-cell lysate preparations. *A,* Western blot analysis of PARP cleavage. *B,* effect of IκBα d.n. mutant and selenium compounds on cleaved PARP: full-length PARP ratio. Amounts of PARP and cleaved PARP presented on Western blots in *A* were quantified using One-Dscan gel and blot analysis software, Scanalytics, Inc. (Fairfax, VA). Ratios of absorbances for bands corresponding to cleaved PARP and full-length PARP are plotted. *Bars,* SD.

κB, the ratio of cleaved PARP:full-length PARP was much strongly affected in those cells than in cells that underwent other treatments (Fig. 9*B*). Thus, both the amounts of cleaved PARP and the ratio cleaved PARP:full-length PARP were much higher in cells where NF-κB activity was inhibited by the expression of IκBα d.n. mutant. These data clearly indicate that NF-κB blockage resulted in sensitization of prostate carcinoma cells to apoptosis induced by selenium compounds.

Discussion

Previous work has shown that selenite and MSeA represent two distinct pools of selenium metabolites (11, 29). Selenite and other selenium compounds that are metabolized to hydrogen selenide (H_2Se) induced DNA single-strand breaks and subsequent cell death by combination of acute lysis and apoptosis in different tumor cell cultures (12). Interestingly, selenite-induced apoptosis does not require caspase activation (12). Thus, less active PARP cleavage in prostate carcinoma cells treated with sodium selenite (Fig. 2*A*) probably reflects this caspase-independent type of apoptosis. MSeA and other immediate precursors of another major selenium metabolite, methylselenol, induced apoptosis mediated by caspase cascades (13) without genotoxicity and necrosis (11). In the work presented here, we have found that at an apoptotic dose, both sodium selenite and MSeA inhibited κB DNA-binding and NF-κB transcriptional activity induced by TNF-α or LPS in two prostate cancer cell lines. Specifically, the two selenium forms showed a differential kinetics of NF-κB inhibitory action; the effect of MSeA was more rapid and transitory, whereas selenite action was slower but persisted much longer. It is important to emphasize that NF-κB suppression by selenite, which exerted a more persistent effect on NF-κB function in comparison with the effect of MSeA, correlated well with the stronger inhibition of cell accumulation by this selenium compound in both cell lines (Fig. 1). Our results obtained in prostate cells are consistent with previous experimental findings that selenite could inhibit κB DNA binding in other mammalian cells (27, 30) and provided further biochemical insights on underlying mechanisms. In our experiments, we showed for the first time that selenium compounds inhibited function of NF-κB transcription factor through the blockage of IKK activity, the kinase complex that phosphorylates IκB inhibitors, and consequently, triggers IκBα degradation. It is important to emphasize that NF-κB blockage by IκBα d.n. mutant sensitized prostate carcinoma cells to apoptosis induced by selenium.

Although our research was focused on IKK as the important step necessary for NF-κB activation, there are other possible direct and indirect mechanisms of selenium effect on NF-κB function. It is known that selenium effects at lower doses are mediated by different selenium-proteins such as glutathione peroxidases, thioredoxin reductases, and others that have broad physiological functions including antioxidant protection and redox regulation of different protein activity (1, 31, 32). At higher doses, selenium can directly exert oxidation of nucleotides, proteins, and cofactors (1). One of the previously proposed mechanisms of the selenium effect on NF-κB activity is such a direct interaction between selenium and NF-κB, which results in adduct formation with the thiol groups of NF-κB proteins and subsequent alteration of NF-κB properties including DNA binding (27). The results of our experiments in cell-free system do not suggest that studied selenium compounds directly inhibit NF-κB DNA binding. Whether this discrepancy is caused by buffer conditions used in the test tube reactions or reflects a specific difference between cell types should be investigated.

How relevant are our findings in terms of cancer chemoprevention or therapeutic intervention by selenium?

(*a*) NF-κB factor activation has been associated with tumorigenesis. NF-κB is constitutively activated in human leukemias and lymphomas as well as in some solid tumors and in cell lines from human solid tumors including breast, ovar-

ian, colon, thyroid, pancreatic, and urinary bladder carcinomas, melanomas, and others (15, 33–38). It was shown that inhibition of NF-κB activity in carcinoma cell lines could dramatically reduce cell growth and metastatic properties *in vivo* (39, 40). Recently, we found that NF-κB is activated in some prostate carcinoma cell lines because of an aberrant activation of IKK, resulting in a high level of IκB-α phosphorylation and turnover (28). These data suggest that constitutive activation of the NF-κB signaling pathway could be an important step during tumor development in prostate. Therefore, selenium inhibition of NF-κB activation during early stages of tumorigenesis, which are likely responsive windows for chemoprevention, could be one mechanism mediating prostate cancer prevention and treatment.

(*b*) NF-κB plays a key role in cell protection against diverse apoptotic stimuli including chemo- and radiotherapeutic treatments through activation of the antiapoptotic gene program in cells (41). Antiapoptotic genes that are regulated by NF-κB include genes encoding Bcl-2-like proteins (A1/Bfl1, Bcl-X_l, and Nr13), inhibitor of apoptosis proteins (H-IAP1, H-IAP2, and X-IAP1), and others (41). It became clear recently that NF-κB is also an important regulator of cell proliferation. There is some evidence that NF-κB proteins are implicated in cell cycle regulation through their effect on the cyclin-dependent kinase/cyclin-dependent kinase inhibitor system (42). The best explored link between NF-κB activation and cell cycle progression involves cyclin D1, a cyclin that is expressed early in the cell cycle and is crucial to commitment to DNA synthesis (43). It was shown that promoters of cyclins D1 and D2 contain κB binding sites and are important transcriptional targets of NF-κB (43, 44). Therefore, inhibition of NF-κB antiapoptotic and proproliferative activities could provide specific and causative links to inhibition of prostate tumor cell growth and survival by selenium compounds. In this regard, it is important to mention that *in vitro* the growth of prostate cancer cells was much more affected by selenium compounds than the growth of primary prostate cells (45).[5]

In conclusion, our study, as a proof of principle, established that an apoptotic dose of selenium exposure can inhibit NF-κB activation through the inhibition of IKK activity and IκB degradation. Because of the involvement of constitutive NF-κB signaling in cancer cell survival and proliferation, our findings suggest that this signal pathway may constitute a molecular target for selenium to exert its anticancer activity. In this regard, it is noteworthy that some other potential cancer preventive natural compounds including green tea polyphenols, phytoestrogen genistein, vitamin E derivatives, silymarin, resveratrol, and curcumin have been also found to be inhibitors of NF-κB and IKK in different cells (46, 47). It will be important to further identify the specific inhibitory mechanisms by each of these diverse agents to build a mechanistic basis for combinatorial application of multiple agents to synergistically inhibit NF-κB signaling to achieve comprehensive prevention of prostate cancer.

[5] I. V. Budunova and Y. J. Yao, unpublished data.

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ORIGINAL ARTICLE

Effects of IKK inhibitor PS1145 on NF-κB function, proliferation, apoptosis and invasion activity in prostate carcinoma cells

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A key antiapoptotic transcription factor, nuclear factor kappa-B (NF-κB), is known to be critically important for tumor cell growth, angiogenesis and development of metastatic lesions. We and others showed previously that NF-κB transcription factor was constitutively activated in androgen-independent prostate carcinoma (PC) cell lines due to the upregulated activity of inhibitor of NF-κB kinases (IKK). In this work, using luciferase assay, electrophoretic mobility shift assay and Northern blot analysis of expression of endogenous κB-responsive genes, we demonstrate that a novel highly specific small-molecule IKK inhibitor, PS1145, efficiently inhibited both basal and induced NF-κB activity in PC cells. We found that PS1145 induced caspase 3/7-dependent apoptosis in PC cells and significantly sensitized PC cells to apoptosis induced by tumor necrosis factor alpha. We also showed that PS1145 inhibited PC cell proliferation. Effects of PS1145 on proliferation and apoptosis correlated with inhibition of interleukin (IL)-6, cyclin D1, D2, inhibitor of apoptosis (IAP)-1 and IAP-2 gene expression and decreased IL-6 protein level. In addition, we found that incubation with PS1145 inhibited the invasion activity of highly invasive PC3-S cells in invasion chamber assay in a dose-dependent manner. Overall, this study provides the framework for development of a novel therapeutic approach targeting NF-κB transcription factor to treat advanced PC.
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Keywords: prostate carcinoma; NF-κB; IKK; apoptosis; invasion

Introduction

One of the contributing factors to high mortality rate from prostate cancer is the extreme resistance of malignant prostate cells to apoptosis induced by radio- and chemotherapy. Thus, the specific induction of apoptosis in prostate carcinoma (PC) cells could play a strategic role for PC treatment.

Nuclear factor kappa-B (NF-κB) transcription factor mediates one of the central signaling pathways, protecting cells from apoptotic death (Karin and Lin, 2002; Kucharczak *et al*., 2003). NF-κB also regulates tumor development through transcriptional regulation of a wide variety of genes that encode antiapoptotic proteins, cell cycle-related proteins, proteins involved in angiogenesis, invasion and metastasis (Ghosh and Karin, 2002; Karin *et al*., 2002; Shishodia and Aggarwal, 2004).

The active NF-κB complex is a homo- or heterodimer composed of proteins from the NF-κB/Rel family: NF-κB1 (p50/105), NF-κB2 (p52/100), RelA (p65), RelB and c-Rel (Verma *et al*., 1995; Baldwin, 1996). In nonstimulated cells, NF-κB resides in the cytoplasm in a complex with the inhibitor protein, collectively called IκB. Several inhibitor of nuclear factor kappa-B (IκB) proteins have been identified, including IκBα, IκBβ and IκBε, and proteins p105 and p100, the precursor molecules for NF-κB proteins p50 and p52, respectively (Verma *et al*., 1995; Whiteside and Israel, 1997). Most agents that activate NF-κB employ a common pathway that involves the phosphorylation of the two N-terminal serines in IκB molecules IκBα, IκBβ, IκBε, and the subsequent ubiquitination and degradation of IκB proteins by the 26S proteasome (Whiteside and Israel, 1997). Signal-induced phosphorylation of IκB is executed by a large 900 kDa IκB kinase (IKK) complex, containing two major IκB kinases (IKK) IKKα and IKKβ, as well as several scaffolding proteins (Karin and Ben-Neriah, 2000).

There is mounting evidence that constitutive NF-κB activation is a common feature of a variety of hematological and solid tumor cell lines and tumors (Rayet and Gelinas, 1999; Karin *et al*., 2002), and that constitutive activation of NF-κB suppresses the susceptibility of tumor cells to apoptosis induced by radio- and chemotherapy. We and others showed previously that NF-κB was constitutively activated in androgen-independent PC cell lines due to the constitutive upregulated activity of IKK kinases (Chen and Sawyers, 2002; Gasparian *et al*., 2002; Suh *et al*., 2002; Zerbini *et al*., 2003). NF-κB inhibition with a IκBα super-repressor in

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PC cells led either to apoptosis or to sensitization to apoptosis induced by tumor necrosis factor alpha (TNF-α) and some other treatments (Herrmann *et al*., 1997; Muenchen *et al*., 2000; Gasparian *et al*., 2002). Most importantly, inhibition of NF-κB with an IκBα super-repressor in PC cells suppressed both growth and development of metastatic lesions by those cells *in vivo* when they were injected into prostate ortotopically (Huang *et al*., 2001). Overall, these data suggest that NF-κB signaling pathway is critically important for PC cell growth and the development of metastases in animal models. Thus, NF-κB represents an important target for PC treatment, especially when NF-κB inhibition is used in combination with other proapoptotic chemotherapeutical drugs.

The results of immunostaining of human PCs for major NF-κB protein RelA/p65 are in line with the data obtained in PC cells *in vitro*. We and others showed that p65 has nuclear localization in a significant number of epithelial cells in prostate tumors, especially in hormone-refractory metastatic PCs (Gasparian *et al*., 2002; Ross *et al*., 2004; Shukla *et al*., 2004; Sweeney *et al*., 2004). Nuclear localization of p65 strongly suggests that NF-κB is activated in human PCs, and that constitutive activation of this key antiapoptotic factor could significantly contribute to the resistance of hormone-refractory PCs to apoptosis induction during chemo- and radiotherapy.

There are several pharmacological approaches to target NF-κB. They include repression of NF-κB transactivation potential, stabilization of IκB inhibitors by proteasome inhibitors and, more recently, inhibition of upstream IKK kinases (Karin, 2004). The unique properties of IKKβ among other serine–threonine kinases allowed successful development of specific IKKβ inhibitors at Millennium Pharmaceuticals Inc. (Hideshima *et al*., 2002; Castro *et al*., 2003; Lam *et al*., 2005) and other companies (Burke *et al*., 2003; Kishore *et al*., 2003; Ziegelbauer *et al*., 2005). In the presented work, we studied the effect of the small-molecule IKK inhibitor PS1145 on the status of NF-κB in PC cells, PC cell growth, sensitivity of PC cells to apoptosis and on the invasion capability of PC cells. We demonstrated that PS1145 efficiently inhibited both basal and induced NF-κB activity in PC cells. PS1145 induced apoptosis in PC cells and significantly sensitized PC cell lines to TNF-α-induced apoptosis in a caspase 3/7-dependent manner. In addition, we found that preincubation with PS1145 inhibited PC cell growth and the invasion activity of highly invasive PC3-S cells in invasion chamber assay.

Results

Expression of activated IKKs in PCs

Our previous studies as well as data by others revealed that androgen-independent PC cells maintain the high level of NF-κB basal activity due to constitutive IKK activation (Gasparian *et al*., 2002). To extend our *in vitro* observations, we performed immunostaining of 10 high-grade PCs (Gleason score 7–9) with Ab against IKKα/β phosphorylated at Ser176/180. It is known that activation of IKKα and IKKβ requires their phosphorylation at those specific serines in the activation loop of IKK kinases (Karin and Ben-Neriah, 2000; Huynh, 2000). As shown in Figure 1, activated IKKα/β were strongly expressed in the cytoplasm of epithelial cells in PCs. This finding suggests that IKK kinases are indeed constitutively active in prostate cells in tumors and correlates well with nuclear localization of p65/RelA and NF-κB activation in PCs previously described in our work and in other publications (Gasparian *et al*., 2002; Ross *et al*., 2004; Shukla *et al*., 2004; Sweeney *et al*., 2004).

PS1145 inhibited basal and induced IκBα phosphorylation and NF-κB activity in androgen-independent PC cell lines

We and others have shown that NF-κB is constitutively activated in androgen-independent PC cell lines due to the constitutive upregulated activity of IKK kinases. The IκBα protein, a key substrate for IKKα/IKKβ kinase complex, is constitutively phosphorylated in PC3 and DU145 cells (Gasparian *et al*., 2002). Despite the high constitutive level of NF-κB activity, androgen-independent PC cells appeared to be highly sensitive to diverse NF-κB inducers (Gasparian *et al*., 2003). For example, DU145 cells are highly responsive to TNF-α and lipopolysaccharide (LPS), while PC3 cells are highly responsive to LPS and TPA, but not to TNF-α. Based on these findings, we selected different NF-κB inducers to study the effect of PS1145 on NF-κB activation in PC3 and DU145 cells.

To evaluate the effect of the IKK inhibitor PS1145 on phosphorylation of IκBα, we performed Western blot analysis using whole-cell proteins from DU145 or PC3 cells treated with PS1145 at concentrations 0.5–20 μM for 2–24 h. We found that the PS1145 effect was especially pronounced when cells were incubated with this IKK inhibitor at concentrations 10–20 μM (Figure 2a and data not shown). The significant decrease of IκBα constitutive phosphorylation was revealed 12–16 h after treatment in both DU145 and PC3 cells (Figure 2a and b and data not shown). Furthermore, IκBα phosphorylation was almost completely blocked when cells were incubated with PS1145 for 24 h (Figure 2a and b). However, in PC3 cells decrease in

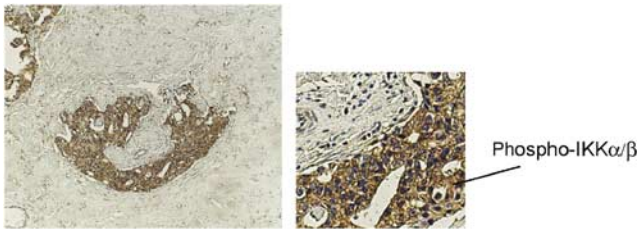

Figure 1 IKKα/β are phosphorylated in PCs. Immunostaining of PC (Gleason score 9) with antibodies against IKKα/β phosphorylated at Ser176/180 in IKK activation loop. Note the strong expression of phosphorylated IKKs in epithelial cells of PC.

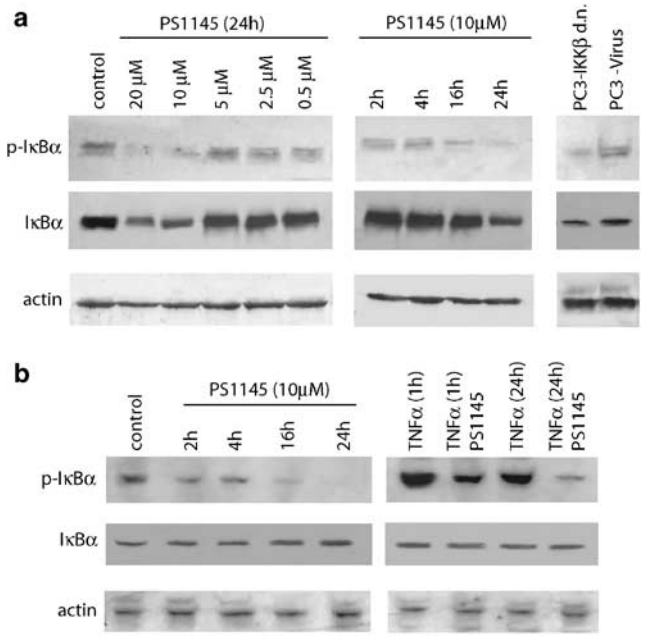

Figure 2 PS1145 inhibited basal and induced IκBα phosphorylation. (**a**) PC3 cells were treated with PS1145 at the indicated concentrations (0.5–20 μM) for 2–24 h. Far right lanes: PC3 cells stably infected with empty lentivirus or lentivirus expressing d.n. IKKβ mutant. (**b**) DU145 cells were treated with 10 μM PS1145 for 2–24 h. Far right lanes: DU145 cells were pretreated with PS1145 for 24 h and treated with TNF-α (7 ng/ml) for 1 h or treated with both agents for 24 h. Western blots containing whole-cell protein extracts (50 μg/lane) were probed with anti-IκBα and anti-phospho-IκBα antibodies or anti-actin Ab as a control.

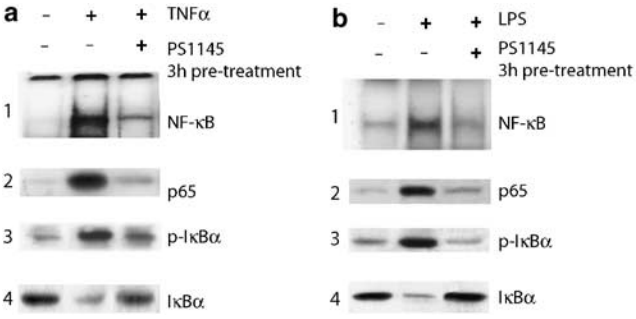

Figure 3 PS1145 inhibited NF-κB binding in PC cells. (**a**). Control and PS1145-pretreated (10 μM × 3 h) DU145 cells were treated with TNF-α (7 ng/ml) for 10 min. (**a.1**) Nuclear protein extracts were used to evaluate κB binding by EMSA; (**a.2**) Western blots containing nuclear protein extracts (20 μg/lane) were probed for expression of p65; (**a.3–a.4**) Western blots containing cytoplasmic protein extracts (20 μg/lane) were probed for expression of IκBα and IκBα-P. (**b**) Control and PS1145-pretreated (10 μM × 3 h) PC3 cells were treated with LPS (1.5 μg/ml) for 1 h. Procedures used in (**b.1–b.4**) are identical to those described in (**a.1–a.4**).

IκBα phosphorylation 24 h after PS1145 treatment coincided with inhibition of total IκBα expression at this time point (see below), suggesting that the relative level of inhibition of IκBα phosphorylation (ratio P-IκBα:total IκBα) in PC3 cells was similar after 16 and 24 h of treatment with PS1145.

PS1145 also strongly inhibited induced IκBα phosphorylation. Indeed, preincubation of PC cells with PS1145 for 3 h significantly inhibited IκBα phosphorylation induced by short treatments with different compounds such as TNF-α, LPS and TPA (Figure 3 and data not shown). In addition, we found a stable strong effect of long cell pretreatment with PS1145 (24 h) on TNF-α-induced IκBα phosphorylation in DU145 cells (Figure 2b). Importantly, the similar decrease in IκBα phosphorylation was observed in PC3 cells stably infected with lentivirus expressing IKKβ dominant-negative (d.n.) mutant (Figure 2a, last lane).

We have to mention that treatment of PC3 cells with PS1145 for 16–24 h as well as the transfection with exogenous IKKβ d.n. mutant has resulted in the decreased level of total IκBα. This reflects the dramatic downregulation of IκBα gene transcription in PC3 cells under this treatment (Figure 5b) and will be discussed later. PS1145 did not affect IκBα expression in DU145 cells (Figure 2b).

In our next experiments, we examined PS1145 effect on NF-κB DNA binding in PC cells. The effect of PS1145 on different steps of NF-κB activation was dose-dependent, and more pronounced when we used PS1145 at the concentration of 20 μM (data not shown). As shown in Figure 3, pretreatment of DU145 cells with PS1145 for 3 h strongly inhibited IκBα phosphorylation, delayed the degradation of IκBα and nuclear translocation of p65 induced by TNF-α, and accordingly significantly decreased the effect of TNF-α on κB DNA binding. Similarly, PS1145 inhibited IκBα phosphorylation, degradation, p65 nuclear translocation and NF-κB binding induced by LPS (Figure 3b) and TPA (data not shown) in PC3 cells.

To evaluate the effect of PS1145 on gene transcription, we used transient transfection of PC cells with a 5 × κB luciferase reporter. To induce NF-κB-dependent transcription of reporter gene, PC cells were transfected with exogenous IKKβ or treated with the appropriate κB inducer: TNF-α (for DU145 cells) or LPS (for PC3 cells) for 24 h. NF-κB activity induced by TNF-α, LPS and exogenous IKKβ was strongly inhibited by PS1145 in both PC cell lines (Figure 4a and b). In addition, PS1145 significantly blocked basal NF-κB transcriptional activity in PC3 cells (Figure 4b).

To study the effect of PS1145 on the transcription of endogenous κB-responsive genes, we used Northern blot analysis of IκBα expression. IκBα gene has five κB sites in its promoter, and is tightly regulated by NF-κB in different cells (Ito *et al*., 1994). We showed previously that the level of steady-state IκBα expression directly correlated with the level of constitutive NF-κB activity in different PC cell lines (Gasparian *et al*., 2002). The results of Northern blotting demonstrated that treatment with PS1145 (20 μM) for 24 h blocked both basal and inducible expression of IκBα in PC cells (Figure 5a and b). The effect of PS1145 on IκBα expression was dose- and time-dependent (data not shown) with maximum IκBα expression blockage after 24 h exposure to PS1145.

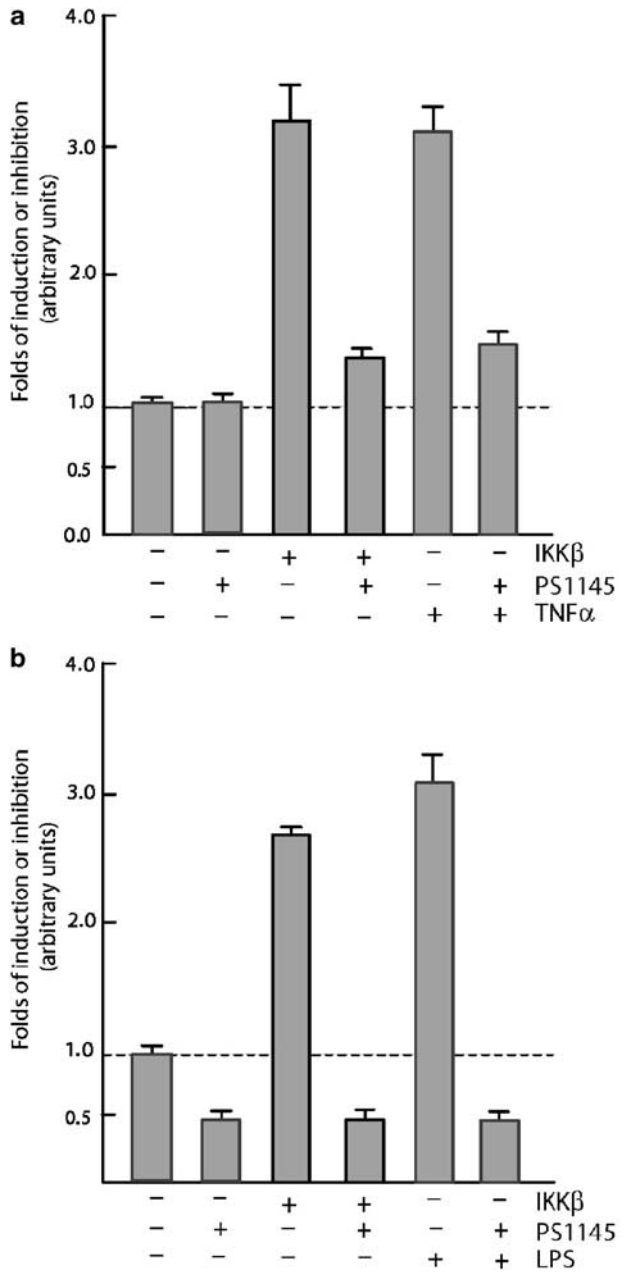

Figure 4 Effect of PS1145 on NF-κB transcriptional activity in PC cell lines. DU145 (**a**) and PC3 (**b**) cells were cotransfected with $\times 5$ κB FL reporter, pRNL-null (RL), pcDNA and with w.t. IKKβ plasmids. Cells were treated with TNF-α (7 ng/ml), LPS (10 μg/ml), PS1145 (10 μM) or the combination PS1145 + TNF-α or PS1145 + LPS for 24 h. Luciferase activity was measured by dual luciferase assay. Data are shown as fold of inhibition or induction, calculated as FL/RL ratio for treated samples normalized against FL/RL ratio for control samples (controls are presented in the far left lanes in (**a**) and (**b**)).

Overall, the results of Northern blot analysis correlated well with the data obtained by Luciferase reporter assay and electrophoretic mobility shift assay (EMSA). We found that PS1145 strongly inhibited NF-κB activity in both PC cell lines irrespectively of the nature of the NF-κB inducer. Notably, the effect of PS1145 on basal NF-κB activity was revealed easier in PC3 cells despite

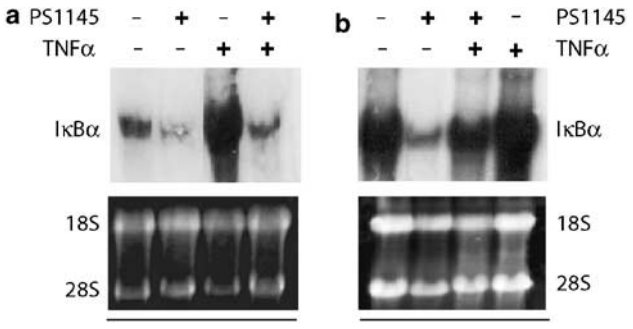

Figure 5 Northern blot analysis of IκBα expression in PC cells after PS1145 treatment. DU1145 (**a**) and PC3 cells (**b**) were treated with TNF-α (7 ng/ml), PS1145 (10 μM) or the combination PS1145 + TNF-α for 24 h. Northern blots (20 μg RNA/lane) were probed for expression of IκBα. Ethidium bromide gel staining (lower panels) was used for the verification of equal RNA loading.

the fact that PS1145 strongly inhibited basal IκBα phosphorylation in both PC cell lines.

PS1145 inhibited proliferation of DU145 cells

The effect of PS1145 on proliferation was assessed by several approaches in DU145 cells. As shown in Figure 6a, MTT test revealed 30–35% decrease in DU145 cell numbers 48–72 h after PS1145 treatment. BrdU labeling of DU145 cells confirmed the result of MTT test. The number of BrdU-positive cells (cells in S-phase) was decreased by $32 \pm 3.25\%$ in DU145 cell cultures treated with PS1145 for 72 h (Figure 6b). The inhibition of proliferation was further confirmed by the decrease of Ki67 protein expression known to be present in cells in G1, S, G2 and M phases, but not in G0 phase of the cell cycle (data not shown). Therefore, we have shown that PS1145 caused significant inhibition of proliferation in DU145 cells.

PS1145 induces apoptosis in DU145 cells and increases their sensitivity to TNF-α

Long-term exposure to PS1145 was toxic for DU145 cells. To evaluate the effect of PS1145 on apoptosis in these cells, we measured caspase 3/7 activity, and used Western blot analysis to assess poly-(ADP-ribose) polypeptide (PARP) cleavage. As shown in Figure 7a, treatment of DU145 cells with PS1145 for 48 h resulted in strong activation of caspase 3/7 in a dose-dependent manner. Western blot analysis of caspase 3/7-dependent PARP cleavage also demonstrated that PS1145 induced apoptosis in DU145 cells 48–72 h after the beginning of the treatment (Figure 7b and c). Importantly, PS1145 sensitized DU145 cells to TNF-α-induced apoptosis: PARP cleavage and caspase 3/7 activation (Figure 7b and c) were much more pronounced in DU145 cells treated with combination of TNF-α and PS1145. These data are in line with the previous observations that NF-κB protects different cells, including PC cells, against apoptosis induced by TNF-α, and that NF-κB blockage by different genetic approaches results in cell sensitization to TNF-α (Muenchen *et al*., 2000; Gasparian *et al*.,

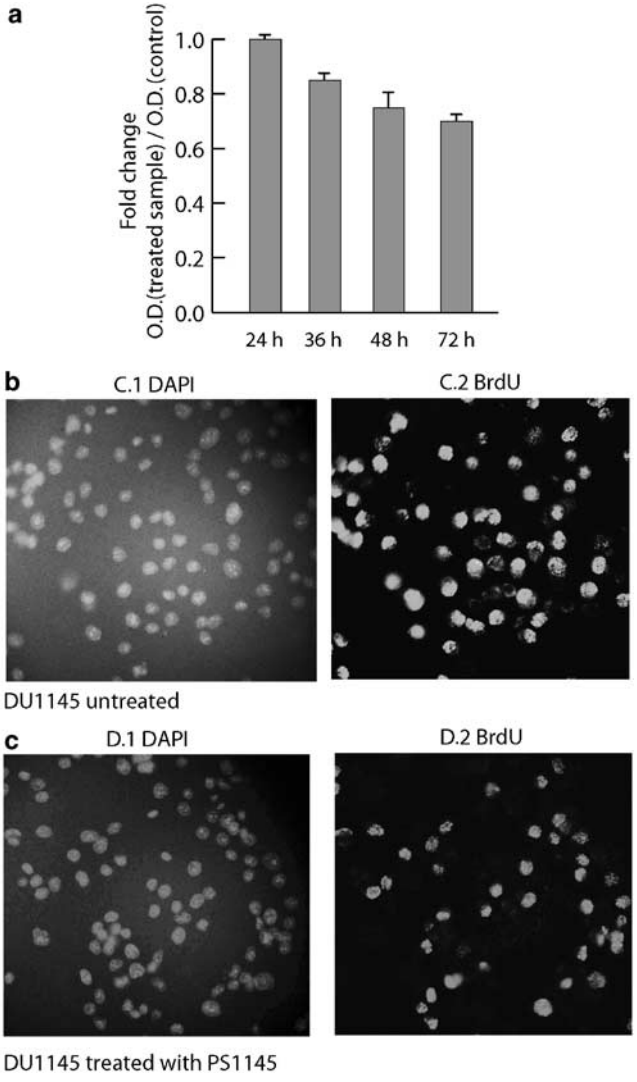

Figure 6 PS1145 inhibited proliferation of DU145 cells. (**a**) DU145 cells were treated with 10 μM PS1145 for the indicated periods of time, and cell growth was evaluated by MTS test. (**b**, **c**) Analysis of BrdU incorporation in DU1145 cells untreated (**b**) and treated with 10 mM PS1145 (**c**). Cells were treated with BrdU for 1 h, fixed in 4% formaldehyde and used for immunofluorescence with anti-BrdU antibody. DAPI nuclear staining and BrdU staining were evaluated by fluorescent microscope, $\times$ 320.

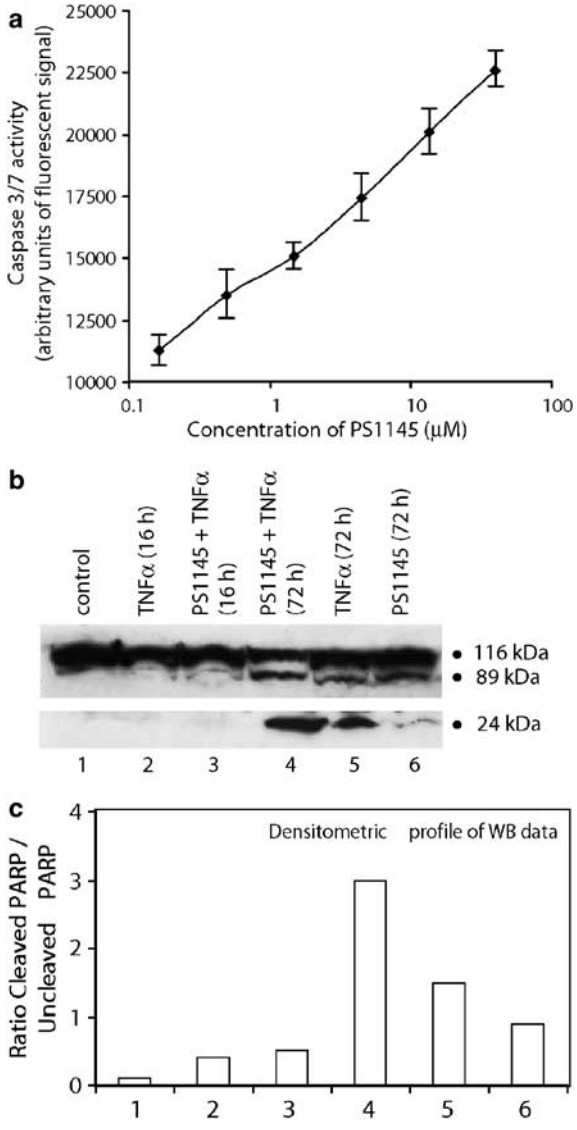

Figure 7 PS1145 induced apoptosis in DU145 cells. (**a**) Caspase 3/7 activity was evaluated in DU145 cells by ApoOne kit after cells were treated with increasing concentrations of PS1145 for 48 h. (**b**) Western Blot analysis of PARP cleavage in DU1145 cells treated with TNF-α (7 ng/ml), PS1145 (10 μM), or the combination PS1145 + TNF-α for 16 and 72 h. (**c**) Densitometric profile of Western blot analysis of PARP cleavage. The data are presented as the ratios of total cleaved PARP products of 89 and 24 kDa to uncleaved PARP.

2002; Orlowski and Baldwin, 2002; Shukla and Gupta, 2004).

Overall, our data indicate that the effect of PS1145 on PC cell growth and apoptosis develops after prolonged treatment (i.e. requires cell maintenance under the conditions when NF-κB is chronically inhibited), even though the significant NF-κB inhibition is achieved in 16 h.

PS1145 inhibited the invasion activity of PC3-S cells in vitro

It is known that DU145 cells do not possess high invasion and migration capability in *in vitro* and *in vivo* experiments. In contrast, several clones derived from the original PC3 cell line were reported to be highly invasive in animals and in invasion chambers *in vitro*. Thus, to study the effect of PS1145 on PC cell motility and invasiveness, we used a highly invasive PC3 clone PC3-S (Lindholm *et al*., 2000). The cell invasiveness was studied using invasion assay of radioactively labeled cells. As shown in Figure 8, PS1145 dramatically inhibited invasion of PC3-S cells in a dose-dependent manner. The effect was more pronounced if the cells were preincubated with PS1145 for 24 h. The inhibition of invasion by PS1145 was not due to PS1145 toxicity for PC3-S cells. PC3-S cells appeared to be rather resistant to the toxic effect of PS1145 evaluated by

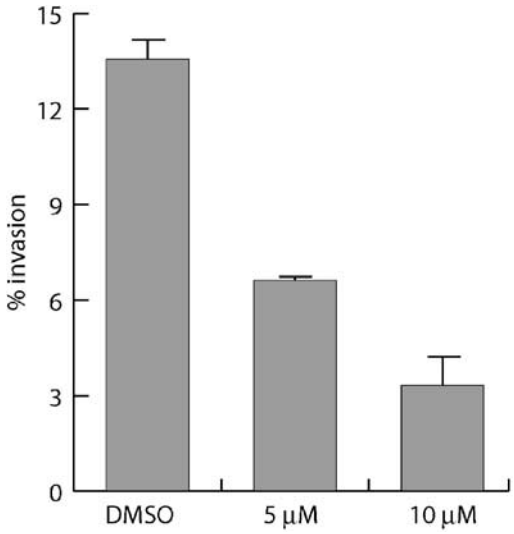

Figure 8 PS1145 inhibited the invasion capacity of PC3-S highly invasive clone. PC3-S cells were pretreated with 5 and 10 μM PS1145 for 24 h and incubated in the Transwell® invasion chamber for up to 72 h. The data are presented as percent of invasion calculated by dividing the invading cell-associated c.p.m. to the total cell-associated c.p.m. (see Materials and methods).

flow-cytometric analysis with propidium iodide (data not shown).

PS1145 effects on gene expression in PC cells

NF-κB regulates a wide variety of genes that encode antiapoptotic proteins, cell cycle proteins, cytokines, proteins involved in cell–cell and cell–extracellular matrix (ECM) interactions and others. Thus, to address the mechanisms underlying the effects of PS1145 on PC cells, we performed semiquantitative RT–PCR analysis of the set of 21 genes known to be important for control of cell cycle, apoptosis and cell–cell and cell–ECM interactions (Toth *et al*., 1995; Glasgow *et al*., 2000; Catz and Johnson, 2001; Hinz *et al*., 2001; Gupta *et al*., 2002; Martone *et al*., 2003). The gene selection was made after the comparison of database obtained by cDNA array analysis of global effect of PS1145 on gene expression in PC cells (these data are not shown in this paper, and are planned to be used for another research project) and the literature database for NF-κB-dependent genes (http://www.nf-kb.org). The list of selected genes is presented in Table 1. The quantitative analysis of gene expression was performed by Agilent 2001 Bioanalyzer as described in Materials and methods.

As shown in Figures 9 and 10, we found that the expression of nine out of 21 studied genes was significantly changed in DU145 cells treated with PS1145, especially after longer 72 h treatment. Figure 9 represents the agarose electrophoresis analysis of RT–PCR products. Figure 10 shows quantitative analysis of expression of all the selected genes (Table 1). As expected, the expression of well-known NF-κB-dependent genes such as inhibitor of apoptosis (IAP)-1, IAP-2, cyclin D1, D2, interleukin (IL)-6 and IL-9 was

Table 1 Genes studied in DU145 and PC3 cells treated with PS1145

Gene group	*Gene name*
1. Apoptosis-related genes	IAP1; IAP2; XAF1; BCL2; BAX; AVEN; c-FLIP, AVEN
2. Proliferation-related genes	Cyclin D1, Cyclin D2, Cyclin B1, Cyclin B2, Cdk4, Cdk6, Cdc2, Cdc5, Cdc6, Cdc25B
3. Angiogenesis-related genes	VEGF-A, VEGF-C, VEGF-D
4. Adhesion molecules	N-cadherin1, ICAM-1
5. Cytokines	IL-6, IL-9

significantly decreased in DU145 cells with inhibited NF-κB activity. The similar inhibition of those genes was found in PC3 cells (data not shown). The expression of three other apoptosis-related genes c-FLIP (CASP8 and FADD-like apoptosis regulator precursor, long isoform), XAF1–X-linked inhibitor of apoptosis protein (XIAP)-associated factor and cell death regulator AVEN was increased in both PC cell cultures, while we did not find any changes in expression of Bcl-2 and Bax. The relevance of those changes to apoptosis induced by PS1145 in PC cells will be discussed below. We also did not find significant changes in the expression of cyclin B1 and B2, and Cdks in PC cells, even though some of those cell cycle-related genes have been previously reported to be downregulated by NF-κB inhibitors (Guttridge *et al*., 1999; Gupta *et al*., 2002). Genes from the vascular endothelial growth factor (VEGF) family were differentially regulated in two PC cell lines: VEGF-C was significantly downregulated only in DU145 cells and VEGF-D was downregulated only in PC3 cells (Figure 10). Overall, the antiapoptotic and antiproliferative effects of PS1145 correlated well with downregulation of IAP-1, IAP-2, cyclin D1 and D2.

It is known that cytokine IL-6 plays an important role in the growth of androgen-independent prostate tumor cells via autocrine and paracrine mechanisms (Giri *et al*., 2001; Zerbini *et al*., 2003; Culig *et al*., 2004). To extend our finding on the inhibition of IL-6 gene expression by PS1145 in DU145 cells, we used ELISA assay to evaluate the amount of IL-6 protein secreted into the cell culture medium by DU145 cells treated with PS1145. As shown in Figure 11, inhibition of IKK resulted in a significant decrease of IL-6 levels.

Effect of PS1145 treatment on cell signaling pathways

The data obtained in our studies indicate that, despite the strong effect of PS1145 on NF-κB activity, its effect on proliferation and apoptosis in PC cells was more modest. This raised the question about the possible activation of some proproliferative, such as mitogen-activated protein kinase (MAPK) (Zerbini *et al*., 2003), and antiapoptotic, such as Akt (Culig *et al*., 2004; Li *et al*., 2005), signaling pathways in PC cells to compensate for NF-κB blockage. To assess the effect of PS1145 on Akt, MAPK and stress-activated protein kinase/Jun-N-terminal kinase (SAPK/JNK) signaling, we evaluated the levels of Akt, SAPK/JNK and dual-specificity mitogen-activated protein kinase kinase 1

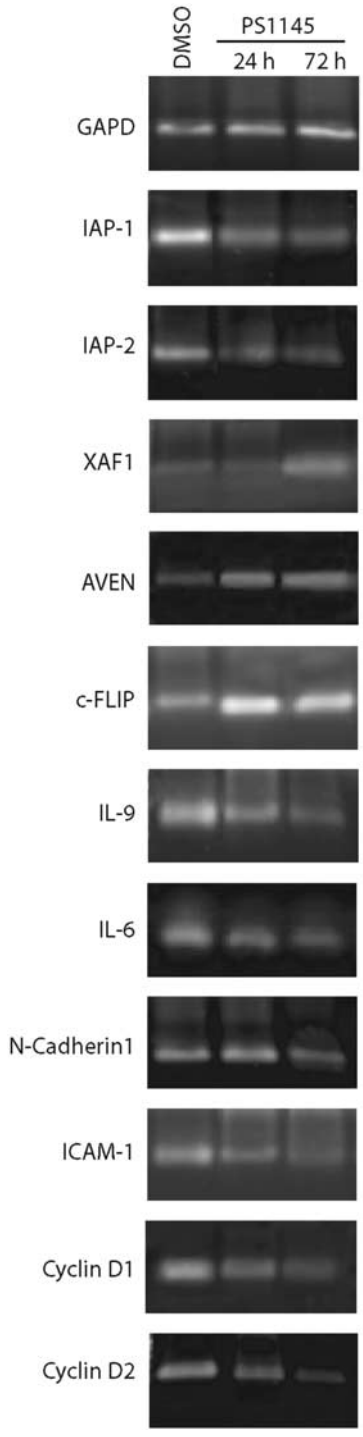

Figure 9 PS1145 effects on the expression of selected genes in DU145 cells. Agarose gel analysis of RT–PCR products of selected genes in DU145 cells treated with 10 μM PS1145 for 24 or 72 h.

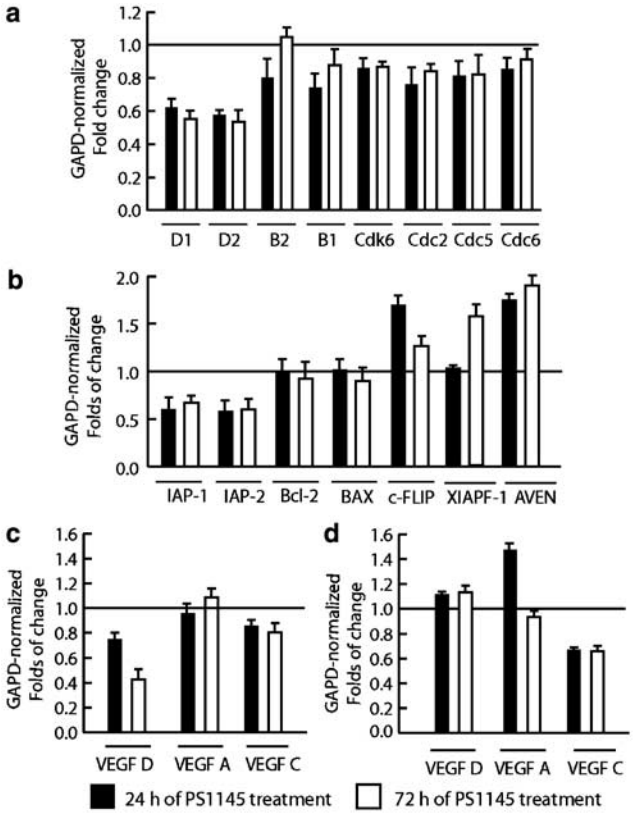

Figure 10 Quantitative analysis of PS1145 effect on gene transcription in DU145 and PC3 cells. RT–PCR products were quantitatively analysed using Agilent 2002 Bioanalyzer. The data are shown as GAPDH-normalized fold change factor calculated for each gene as GAPD-normalized amount of RT–PCR product from PS1145-treated DU145 cells divided by that of untreated DU145 sample. (**a**) Analysis of expression of cell cycle-related genes in DU145 cells. (**b**) Analysis of apoptosis-related genes in DU145 cells. (**c**) Expression of angiogenesis-related genes in PC3 cells. (**d**) Expression of angiogenesis-related genes in DU145 cells.

(Mek1/2) phosphorylation in DU145 cells treated with PS1145 for 24 and 72 h. As shown in the Figure 12, there was no change in the phosphorylation level of SAPK/JNK kinases. Against expectations, Akt activity was inhibited by long, 72-h treatment of DU145 cells with PS1145. Interestingly, phosphorylation of c-Raf and downstream Mek1/2 was strongly, even though temporarily, increased in response to PS1145 treatment. This correlated very well with increased phosphorylation of downstream Mek1/2 target kinases extracellular signal-regulated kinase (Erk)1/2, that was mostly pronounced 24 h after treatment with PS1145.

Discussion

There is mounting evidence that NF-κB activation is associated with tumorigenesis. NF-κB was found to be activated in human leukemias and lymphomas, lung and breast carcinomas, as well as in numerous cell lines of different origin (Rayet and Gelinas, 1999; Karin *et al*., 2002). The chronic activation of NF-κB in tumor cells has been linked both to genetic changes and to epigenetic mechanisms. There are numerous reports indicating that upstream signaling pathways causing (or associated with) tumor development can activate NF-κB. Viral oncoproteins including Tax and EVB nuclear antigen are known to activate NF-κB through

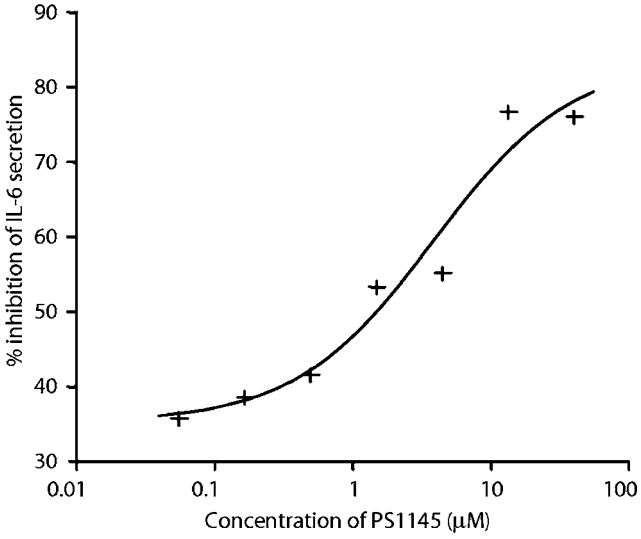

Figure 11 Analysis of PS1145 effect on IL-6 protein expression in DU145 cells. Cell culture medium was harvested 48 h after treatment of DU145 cells with PS1145 at increasing concentrations, and IL-6 expression was evaluated in cell culture medium by ELISA (Quantikine assay).

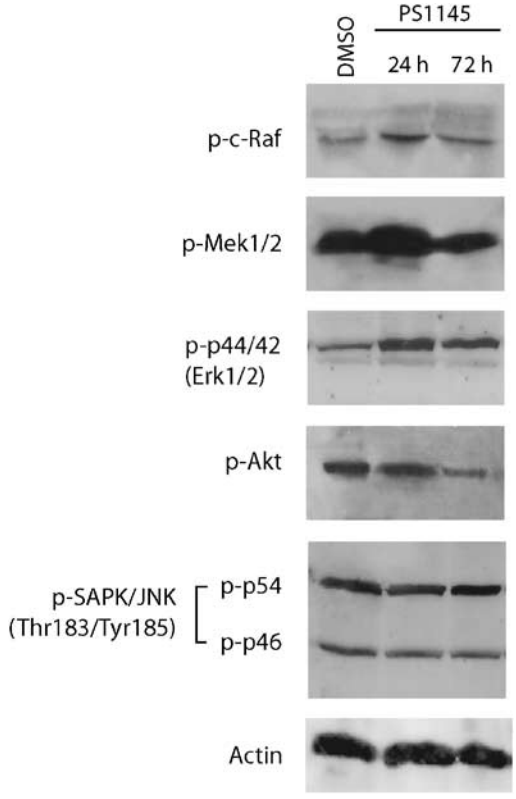

Figure 12 Analysis of PS1145 effect on cell signaling pathways. Western blot analysis of whole-cell protein extracts (50 μg/well) prepared from DU145 cells treated with 10 μM PS1145 for the indicated periods of time. Western blot membranes were probed for expression of phosphorylated c-Raf, Mek1/2, Erk1/2, SAPK/JNK, Akt and actin as a control for loading.

interaction with IKK complex or some other mechanisms (Karin *et al*., 2002; Orlowski and Baldwin, 2002). NF-κB and IKK complex could be induced by activated oncogens Ras, Bcr–Abl, members of Rho protein family (Orlowski and Baldwin, 2002). Production of numerous growth factors and cytokines that are strong activators of IKK complex, and whose expression is in turn NF-κB-dependent, is typical for tumor cells (Orlowski and Baldwin, 2002; Zerbini *et al*., 2003; Greten and Karin, 2004). Those cytokines, including IL-6 and growth factors, may contribute to the establishment of positive autocrine/paracrine loops of NF-κB activation in tumor cells (Giri *et al*., 2001; Zerbini *et al*., 2003; Culig *et al*., 2004). There is also evidence that IKK-independent pathways, including p65 phosphorylation, can be involved in NF-κB constitutive activation in tumor cells (Viatour *et al*., 2005).

Recently, we and others found that NF-κB is activated in androgen-independent PC cells and in prostate tumors, where NF-κB has nuclear localization in at least 15% of cells (Palayoor *et al*., 1999; Gasparian *et al*., 2002; Ross *et al*., 2004; Shukla *et al*., 2004; Sweeney *et al*., 2004). The major mechanism of NF-κB activation in PC cell lines involves the aberrant activation of IKK complex, resulting in increased phosphorylation and instability of IκB inhibitor proteins (Gasparian *et al*., 2002). Importantly, in this work using immunostaining with antibodies against activated, phosphorylated IKKs, we showed for the first time that IKK complex is also activated in PC samples. Thus, IKKs, especially IKKβ that is critical for NF-κB activation, represent a novel important target for NF-κB blockage in PC and other tumor cells. Very recently, several pharmaceutical companies have started working on the design of potent orally active IKKβ inhibitors (Burke *et al*., 2003; Kishore *et al*., 2003; Baxter *et al*., 2004; Murata *et al*., 2004; Ziegelbauer *et al*., 2005). PS1145 is one of these highly specific IKK inhibitors (IC $<$ 0.1 μM) recently developed by Millenium Pharmaceuticals, Inc. (Hideshima *et al*., 2002; Lam *et al*., 2005).

In this work, we developed a comprehensive picture of the effects of PS1145 on NF-κB activity, growth, sensitivity to apoptosis and invasiveness of PC cells. We first demonstrated that pretreatment with PS1145 (10–20 μM) efficiently inhibited both basal and induced NF-κB activity in PC cells. Then we studied the major biological responses of PC cells resulting from NF-κB inhibition. We showed that PS1145 inhibited proliferation of DU145 cells when cells were incubated with IKK inhibitor for 48–72 h. These data are in line with the previous findings indicating that NF-κB is an important regulator of cell proliferation, and its effect is mediated through regulation of expression of cyclins (especially cyclin D1), possibly CDK/CKI genes, and some other cell cycle-related genes, for example, c-*myc*. Our study has revealed that PS1145 significantly inhibited cyclin D1 and D2 expression in PC cells, but did not affect the expression of cyclin B1, B2, cdk6, cdk4, cdc2, cdc5 and cdc6. Relatively modest inhibition of PC cell proliferation by PS1145 correlates well with the results obtained

in multiple myeloma cells treated with the same IKK inhibitor (Hideshima *et al*., 2002), and in PC-3 cells transfected with IκBα super-repressor (Huang *et al*., 2001), and may reflect the existence of compensatory mechanisms that counteract NF-κB blockage in PC cells. Indeed, we found that Raf/Mek1/2/Erk1/2 kinases were strongly activated in DU145 cells by PS1145. This suggests that simultaneous inhibition of NF-κB and MAP kinase cascade may result in more profound inhibition of PC cell proliferation.

A key role of NF-κB in cell protection against diverse apoptotic stimuli including chemo- and radiotherapy is very well known. The antiapoptotic NF-κB-regulated genes include genes that encode Bcl-2-like proteins (A1/Bfl1, Bcl-X_l and Nr13), IAP proteins – IAP-1, IAP-2, X-IAP-1 and others (Barkett and Gilmore, 1999). In our experiments, PS1145 itself triggered modest apoptosis in DU145 cells treated for 72 h or longer. Most importantly, PS1145 treatment significantly sensitized relatively resistant DU145 cells to TNFα-induced apoptosis. These data correlate well with the previous findings that NF-κB blockage by overexpression of nondegradable IκBα mutant may result in apoptosis or in sensitization to TNF-α-induced apoptosis in PC cells. Further analysis of mechanisms of apoptotic death induced by PS1145 in PC cells revealed the central role of caspase 3/7 in this process. Indeed, we found that PS1145 induced caspase 3/7 activation and, consequently, increased cleavage of PARP, a target protein for caspase 3/7. In turn, the activation of caspase 3/7 correlated with the decreased expression of its inhibitors, IAP-1 and IAP-2 after PS1145 treatment. Moreover, our data indicated the role of XIAP-associated factor-1 (XAF1), an antagonist of another inhibitor of caspase 3/7, X-IAP. We found that XIAF1 expression was significantly increased in PC cells treated with PS1145. On the contrary, we did not find changes in the expression of genes involved in mitochondrial apoptosis (Abraham and Shaham, 2004; Rapp *et al*., 2004). Unexpectedly, certain antiapoptotic genes have been activated in PC cells after PS1145 treatment. For example, the expression of genes that encode caspase 8 (FLICE) inhibitory protein c-FLIP (long FLIP isoform), and especially cell death regulator Aven was increased in DU145 cells treated with PS1145. Aven was recently shown to bind both Bcl-x(L) and the caspase 9 regulator Apaf-1, thus inhibiting mitochondrial apoptosis (Chau *et al*., 2000; Figueroa *et al*., 2004; Peter, 2004). Interestingly, the effect of PS1145 on Aven expression was especially pronounced in PC3 cells more resistant to PS1145-induced apoptosis, than in DU145 cells (data not shown). This may potentially explain the known higher resistance of PC3 cells to apoptosis induced by NF-κB blockage.

As we mentioned, the specific pharmacological IKK inhibitors have been developed only recently. Thus, the information about their effect on tumor cell behavior is very limited. PS1145 was recently tested in multiple myeloma cells (Hideshima *et al*., 2002). Another novel IKK inhibitor, BMS-345541 (Burke *et al*., 2003), was studied in human melanoma cells (Yasui *et al*., 2003). Overall, the effects of IKK inhibitors in other tumor cells were similar to our findings in PC cells. Both IKK inhibitors decreased tumor cell proliferation *in vitro*, and either induced apoptosis or sensitized tumor cells to apoptosis induced by TNF-α. BMS-345541 also inhibited melanoma cell growth *in vivo* as xenografts in nude mice. Interestingly, in both cell types, IKK inhibition has resulted in abrogation of paracrine growth loops, mediated in multiple myeloma cells by IL-6, and in melanoma cells by chemokine CXCL1. Aberrant expression of IL-6 has been implicated in PC progression and resistance to chemotherapy (Giri *et al*., 2001; Zerbini *et al*., 2003; Culig *et al*., 2004). IL-6 is highly expressed in androgen-independent PC cell lines, and has been shown to function as an important growth factor in PC cells, possibly also through autocrine growth loop (Zerbini *et al*., 2003). Importantly, treatment of PC cells with PS1145 resulted in significant decrease of IL-6 gene expression and decreased concentration of IL-6 protein in cell culture medium, suggesting that PS1145 affects positive growth loop mediated by IL-6 in PC cells.

In conclusion, the presented results obtained in PC cell cultures suggest that constitutively active antiapoptotic and proproliferative NF-κB signaling represents a rational target for PC treatment, especially in combination with some other proapoptotic chemotherapeutical drugs. The development of IKK inhibitors that more specifically block NF-κB signaling than all other agents including proteasome inhibitors will be very helpful to block NF-κB as a novel anticancer strategy in clinics.

Materials and methods

Cell cultures and treatments

DU145 and PC3 cells were purchased from the American Tissue Culture Collection (Rockville, MD, USA). DU145 and PC3 cells were cultured in RPMI 1640 medium (Gibco BRL Life Technologies, Rockville, MD, USA) supplemented with 10% FBS (HyClone, Logan, UT, USA), sodium pyruvate (1 mM, Sigma Chemical Co., St Louis, MO, USA) and antibiotics (Gibco BRL Life Technologies, Rockville, MD, USA). The following reagents were used for cell treatments: PS1145 (Millenium Pharmaceuticals, Inc., Boston, MA, USA), LPS, DMSO and TNF-α from R&D Systems (Minneapolis, MN, USA). PS1145 was dissolved in DMSO, and stock solution was stored at −20°C. PC3 cells stably expressing d.n. IKKβ mutant tagged with FLAG were generated using lentivirus system from Invitrogen Corporation (Carlsbad, CA, USA) following the manufacturer's protocol. Antibiotic blasticidin was used at concentration of 6 μg/μl to select for d.n. IKKβ-expressing clones. The d.n. IKKβ expression was confirmed using anti-FLAG antibodies (Sigma Chemical Co., St Louis, MO, USA) and anti-IKKα/β antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) by Western blotting and immunostaining (data not shown). We used in our work pulled PC3-d.n. IKKβ cell cultures.

Preparation of cellular extracts and electrophoretic mobility shift assay

Nuclear and cytosolic proteins were isolated as described previously (Lyakh *et al*., 2000). The binding reaction for

EMSA contained 10 mM HEPES (pH 7.5), 80 mM KCl, 1 mM EDTA, 1 mM EGTA, 6% glycerol, 0.5 μg of poly(dI-dC), 0.5 μg of sonicated salmon sperm DNA, γ-^{32}P-labeled ($2–3 \times 10^5$ c.p.m.) double-stranded κB-consensus oligonucleotide (Promega Corp., Madison, WI, USA), γ-^{32}P-labeled ($2–3 \times 10^5$ c.p.m.) and 5–10 μg of the nuclear extract. DNA-binding reaction was performed at room temperature for 30 min in a final volume of 20 μl. DNA–protein complexes were analysed on 6% DNA retardation gels (Novex, Carlsbad, CA, USA). Dried gels were subjected to radiography.

Western blot analysis

Whole-cell protein extracts were prepared using RIPA buffer as described elsewhere (Rosenberg, 1996). Proteins were resolved by electrophoresis on 10% SDS–PAGE and transferred to Immobilon-P membrane (Millipore Corporation, Bedford, MA, USA). Anti-phospho-Mek1/2, anti-phospho-Erk1/2, anti-phospho-Akt, anti-phospho-SAPK/JNK, anti-phospho-c-Raf and anti-phospho-Ser32 IκBα and anti-PARP Abs were from Cell Signaling Technology, Inc. (Beverly, MA, USA); anti-p65 and anti-IκBα antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Membranes were blocked with 5% non-fat milk in TBST buffer and incubated with primary antibodies overnight at 4°C. Peroxidase-conjugated anti-rabbit or anti-mouse IgG (Sigma Chemical Co., St Louis, MO, USA) were used as secondary antibodies. ECL Western blotting reagent (Amersham Pharmacia Biotech, Sweden) was used for protein detection. To verify for equal amounts of proteins loaded and transferred, the membranes were stained with Ponceau Red.

Transfection of PC cells and luciferase activity

The following constructs were used for transfections: κB-luciferase reporter – *Firefly* luciferase (FL) under a 5× κB promoter kindly provided by Dr WC Greene (Gladstone Institute for Virology and Immunology, University of California, San Francisco, CA, USA); pRL-null construct – *Renilla* luciferase (RL) under a minimal promoter (Promega Corp., San Luis Obispo, CA, USA); pcDNA3.1-CMV-IKKβ wild type (w.t.) (kindly provided by Dr F Mercurio, Signal Pharmaceutical, Inc., San Diego, CA, USA). PC cells were plated onto 12-well plates and at 50% confluency were cotransfected with indicated plasmids using Effectene reagent (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. The amount of each plasmid DNA was 0.5 μg/well. Prostate cells were harvested 36 h after the transfection and the Luciferase activity was measured by dual luciferase assay (Promega Corp., San Luis Obispo, CA, USA) on a TD20/20 Turner luminometer (Turner Design Instruments, Sunnyvale, CA, USA). When necessary, before transfections, cells were pretreated with PS1145 (10 μM) and/or TNF-α (7 ng/ml) or LPS (1.5 μg/ml). FL activity was normalized against RL activity to equalize for transfection efficacy.

Northern blot analysis

Total RNA from freshly harvested cells was isolated by TRI reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) and subjected to Northern blotting. In all, 20 μg of total RNA was resolved in 1% agarose−6% formaldehyde gel. The RNA was transferred to nylon membranes and probed for IκBα. The DNA probe was prepared by random-primed reactions using the complete coding sequence of human IκBα cDNA (ATCC, Rockville, MD, USA).

RT–PCR

Two-step RT–PCR reaction using reverse transcriptase MLV-RT with random primers and PCR-Supermix (both from Invitrogen Corp., Carlsbad, CA, USA) with appropriate PCR primers was performed using total RNA isolated by RNAeasy mini kit (Qiagen Inc., Valencia, CA, USA). PCR primers were designed using Primer-Bank database (http://pga.mgh.harvard.edu/primerbank/) and RTPrimerDB Real Time PCR Primer and Probe Database (http://medgen.ugent.be/rtprimerdb/index.php). PCR products were analysed by electrophoresis in 1.5% agarose gel. The actual amount of PCR product was measured by an Agilent 2001 Bioanalyzer and normalized to the amount of GAPD PCR product. For quantitative analysis, the data are represented as the ratio of GAPD-normalized amount of PCR product in PS1145-treated cells to the GAPD-normalized amount of PCR product in control cells.

IL-6 secretion

DU145 cells were plated at 5000 cells/well in BD356640 poly-D lysine 96-well plates (Beckton Dickenson, Franklin Lakes, NJ, USA). Cells were incubated with PS1145 at increasing concentrations for 48 h. IL-6 level in tissue culture media was measured using Quantikine Assay for human IL-6 (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol using Wallace Victor 2 1420 luminometer.

Assessment of proliferation

Cell proliferation was assessed using None-Radioactive Cell Proliferation Assay (MTS test) from Promega Corporation (San Luis Obispo, CA, USA) and bromodeoxyuridine (BrdU) cell labeling using immunofluorescence. For both tests PC were plated onto 12-well plates (20×10^3 cells/well), and cultured for 24–72 h in the presence of 10 μM PS1145. Every 24 h fresh complete media containing 10 μM PS1145 was added to the cells. For MTS test each group of cells was plated in triplicate. The MTS reagent was prepared and used according to the manufacturer's protocol. Optical density of the samples was measured on a plate reader at 490 nm.

For BrdU labeling, the cells were plated onto sterilized coverslips placed on the well bottoms in 12-well plates. PS1145-pretreated and control DU145 cell cultures were treated with BrdU (2 μg/ml) for 2 h. The coverslips with cells were fixed and permeabilized with acetone–methanol (1:1) mixture at −20° for 15 min, washed with PBS, blocked with 20% goat serum and stained with primary anti-BrdU Ab from Becton Dickinson (Franklin Lakes, NJ, USA) and secondary goat anti-mouse Ab conjugated with Cy3 (Jackson Immunoresearch laboratories Inc., West Grove, PA, USA). DAPI (Vector laboratories, Burlingame, CA, USA) was used to counterstain the nuclei. The number of BrdU-positive cells was counted in PS1145-treated and control cultures (10 fields of view in each sample). For quantitative analysis, the data were presented as the % of BrdU-positive nuclei to the total number of nuclei stained with DAPI.

Apoptosis detection

To evaluate apoptosis, we used Western blot analysis of PARP proteolysis and caspase 3/7 functional assay. To study PARP proteolysys, prostate cells, plated on 10 mm dishes, were treated with 10 μM PS1145 alone or in combination with 7.5 ng/ml TNF-α (R&D Systems, Minneapolis, MN, USA) for 16–72 h upon reaching 50% confluence. Adherent cells and detached floaters were combined for whole-cell protein extract preparations. PARP cleavage was estimated by Western blot analysis with anti-PARP antibody (Pharmingen, San Diego, CA, USA).

For caspase 3/7 functional assay, DU145 cells were plated at 5×10^3 cells/well of a 24-well plate in 100 μl of complete media. PS1145 was added at increasing concentrations from 0.1 to 50 μM. Caspase activity was measured after 48 h treatment with PS1145 using ApoOne kit (Promega Corp., Madison, WI, USA) according to the manufacturer's protocol. In all, 100 μl of the substrate was added for 2.5 h.

Transwell® invasion assays

The PC-3 High Invasion subclone (PC3-S) was previously selected by serial passages through reconstituted basement membrane Matrigel® (Becton Dickinson, Lincoln Park, NJ, USA) in the Transwell® invasion apparatus (Lindholm *et al*., 2000). For analysis of PC-3S cell invasion in the presence of PS1145 inhibitor, a Transwell® invasion assay was used according to the protocol described previously (Lindholm *et al*., 2000). Prior to the invasion assay, the cells were preincubated with [^{3}H]thymidine and either PS1145 or control vehicle (Lindholm *et al*., 2000) overnight. The cells were incubated in the invasion chamber for up to 72 h. Invading cells were collected as described previously (Lindholm *et al*., 2000), and the cell invasion was quantitated by counting the cell-associated c.p.m. The percent invasion was calculated by dividing the invading cell-associated c.p.m. to the total cell-associated c.p.m. The statistical analysis of percent invasion was determined by Student's *t*-test comparisons using InStat™ statistical software (GraphPad Software, Inc., San Diego, CA, USA). The results are presented as mean $\pm$ standard deviation (s.d.).

Immunostaining of prostate tissues

Prostate tissues were obtained from white male patients at the age 40–82 years during biopsy or surgery to remove prostate tumors. Paraffin sections of formalin-fixed PC samples with verified diagnosis were used for immunostaining. The immunostaining was performed using Envision+ System-HRP (DAB) kit according to the manufacturer's protocol (DakoCytomation, Carpenteria, CA, USA). After Ag retrieval in a pressure cooker (for 5 min at 20–25 psi) in citric buffer (pH 6.0), tissue sections were blocked with 20% goat serum in PBS, and consequently incubated with primary rabbit polyclonal Ab against phospho-IKKα/β (Cell Signaling Technology, Inc., Beverly, MA, USA), followed by secondary anti-rabbit IgG-reagent provided with the Envision+ System-HRP (DAB) kit. Immunostaining was visualized with DAB chromogen (DakoCytomation, Carpenteria, CA, USA) and counterstained in Mayer's hematoxylin.

Data in all figures are shown as results of the representative experiments. All experiments were repeated at least three times.

Abbreviations

EMSA, electrophoretic mobility shift assay; Erk, extracellular signal-regulated kinase; IAP, inhibitor of apoptosis; IKK, inhibitor of nuclear factor kappa-B kinase; IκB, inhibitor of nuclear factor kappa-B; IL, interleukin; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; Mek1/2, dual-specificity mitogen-activated protein kinase kinase 1; NF-κB, nuclear factor kappa-B; PC, prostate carcinoma; SAPK/JNK, stress-activated protein kinase/Jun-N-terminal kinase; TNF-α, tumor necrosis factor alpha.

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mimosine in the late G1 phase of the cell cycle [Krude, T, *Exp Cell Res* 1999, 247, 148–159], as the result of the *in vitro* incubation with cytosolic extracts prepared from asynchronously proliferating or S-phase human cells [Krude, T, *J.Biol.Chem*, 2000, 275,18,13699–13707]. Based on this principle, a homogenous cell-free screening assay has been developed. Nuclei from mimosine-arrested HeLa cells are incubated in buffer, containing dNTPs, Cy5-dUTP, NTPs and an energy regenerating system with or without cytosolic extracts. The nuclei dye SYTO 16 is used for identification of nuclei in 96-well plates and the incorporation of the labeled nucleotide, Cy5-dUTP, allows assessment of the initiation of DNA replication. Plates are imaged using a novel confocal cell scanner platform – The LEADseeker Cell Analysis System, capable of simultaneous multi-wavelength image acquisition and analysis in a high-throughput format. Z factor, a statistical parameter for validation of high throughput screening assays, was shown to be 0.5 in three separate representative experiments (n=10–24), confirming applicability of this assay for screening purposes. Using this assay, we have demonstrated the initiation of DNA replication *in vitro* in nuclei from HeLa cells, synchronised in late- but not early-G1 phase of cell cycle in the presence of cytosolic extracts from asynchronously proliferating HeLa cells (79.12±2.97% of the late G1- and only 7.72±1.81% of the early G1-phase nuclei incorporated Cy5-dUTP when incubated with cytosol, n=4). We were also able to show the dose-dependent inhibition of initiation of DNA replication by known cdk-inhibitor roscovitine and the inhibition of elongation by S-phase inhibitor aphidicolin.

We present here a unique cell-free screening assay, which allows the rapid evaluation of potential inhibitors or stimulants of the initiation of DNA replication.

LB-32 Chemopreventive properties of Selenium compounds are associated with inhibition of IκB kinase and transcriptional factor NF-κB. Gasparian Alexander V., Yao Ya Juan, Lu Junxuan, Slaga Thomas J., and Budunova Irina V. *AMC Cancer Research Center, Denver , CO*

Se compounds are known inhibitors of carcinogenesis in different organs in animal models, and have already shown a great potential as effective preventive agents for prostate cancer. There are several proposed mechanisms of their anticarcinogenic effect, including antioxidant protection and enhanced apoptosis of transformed cells. Because transcription factor NF-κB is an important mediator of oxidative damage, is a key anti-apoptotic factor in mammalian cells, and is involved in oncogenesis, we tested whether Se could modulate NF-κB activity in prostate cells. The key to NF-κB regulation is the inhibitory κB (IκB) proteins that in response to diverse stimuli are rapidly phosphorylated by IκB kinase (IKK) complex, ubiquitinated and degraded releasing NF-κB factor. Thus, we investigated the effect of two Se compounds on NF-κB and IKK activation. In our experiments we studied sodium selenite (Na_2SeO_3) and a novel synthetic compound methylseleninic acid (MeSeA). Na_2SeO_3 is metabolized predominantly to hydrogen selenide and possesses both apoptotic and genotoxic activities in different cell cultures. MeSeA is metabolized predominantly to methylselenol, the putative critical anticarcinogenic metabolite of Se, and induces growth inhibition and apoptosis without genotoxicity.

We found that both Na_2SeO_3 and MeSeA at concentrations of 2.5 – 5 μM inhibited IKK activation induced by TNF-α and LPS and inhibited induced IκB-α phosphorylation and degradation in prostate cells. They also inhibited DNA κB binding induced by TNF-α and LPS. The kinetics of NF-κB inhibition was different with quick and transient inhibition of κB binding by MeSeA and slower but much more sustained inhibition by Na_2SeO_3. Se compounds did not inhibit effect of TNF-α and LPS on other transcription factors. Both compounds inhibited basal and induced κB.Luciferase reporter activity in prostate cell lines. Concentrations of Se that inhibited NF-κB also strongly inhibited growth of prostate cells measured as inhibition of cell proliferation and induction of cell death. These results suggest that Se chemopreventive effect in prostate could be mediated at least partially by inhibiting NF-κB, and emphasize the potential role of IKK as a target of choice to specifically inhibit NF-κB activity in prostate cells.

LB-33 Anti-tumor and mechanistic studies of pachymic acid, a natural triterpenoid from *Poria cocos*. Jifu Zhao, Qiang Sun, Loretta Zapp, and Thomas J. Slaga, *Center for Cancer Causation and Prevention, AMC Cancer Research Center, Denver, CO, and Shenyang Medical College, Shenyang, China*

Pachymic acid (PA), a natural lanostane type-triterpene acid from *Poria cocos* commonly used as Traditional Chinese Medicine, has been shown by Japanese and Chinese investigators to have potent anti-skin tumor promoting effect in mice. In this study, PA was isolated from *Poria cocos* and identified by physiochemical properties and spectroscopic evidences. Initially, the biochemical and molecular mechanisms of PA's effect on tumor promotion in mouse skin were investigated. A single topical application of PA at 1 and 3 mg doses on to the SENCAR mouse dorsal skin was followed 30 min later with a 3 μg dose of 12-O-tetradecanoylphorbol 13-acetate (TPA). This resulted in a 44% ($p<0.05$) and 91% ($p<0.001$) inhibition of TPA-caused skin edema, respectively. TPA-inducible interleukin 1 alpha (IL 1α), myeloperoxidase (MPO) and ornithine decarboxylase (ODC) were also measured using the SENCAR mouse skin model. PA showed a dose-dependent inhibition of the expression of three biomarkers. At the 3 mg PA dose, the expression of IL 1α, MPO and ODC were inhibited by 92% ($p<0.001$), 97% ($p<0.001$) and 87% ($p<0.001$), respectively. Western immunoblot analysis showed that 1 mg and 3 mg doses of PA significantly inhibit TPA-induced epidermal cyclooxygenase-2 (COX-2) by 28% ($p<0.05$) and 62% ($p<0.01$), respectively, with no change in constitutive COX-1 protein levels. The results from above studies suggest that PA might inhibit tumor promoting through its strong anti-inflammatory effect. Secondly, we examined the tumor-therapeutic activity of PA. Mice were inoculated for 20 days with 25 mg/kg PA post subcutaneous injection of Sarcoma 180 cells. PA-treated mice demonstrated inhibition of tumor growth by 63% ($p<0.01$) over control. Additionally, Ehrlich ascites carcinoma-bearing mice were evaluated after 20 days inoculation of 25 mg/kg PA and were found to live 36 % ($p<0.05$) longer than the control group. PA showed much less toxicity compared to that of mice treated with cyclophosphamide, a common cancer chemotheraphy agent. These data encourage the further study of PA on tumor prevention and therapeutic effectiveness.

LB-34 Systemic delivery of a replicating vaccinia expressing purine nucleoside phosphorylase (PNP) treats subcutaneous tumors. Chang, Eugene, Puhlmann, Markus, Alexander, H. Richard, Bartlett, David L.; *Surgery Branch, National Cancer Institute, National Institutes of Health,*

Introduction: A thymidine kinase deleted vaccinia (WR strain) selectively replicates and expresses genes in tumors when delivered systemically in animal models. While we have previously demonstrated an antitumor effect based on replicative cytopathogenicity within the tumor, we believe that this response will be enhanced with an enzyme/prodrug system. We constructed a vaccinia virus encoding for PNP (Vac-PNP) which converts purine analogs into freely diffusable metabolites that are highly toxic to both dividing and non-dividing cells. We have previously reported on the efficacy of Vac-PNP combined with 6MPDR; we now report on Vac-PNP combined with fludarabine which is commercially available and although known to be an effective drug for hematologic malignancies, it has no effect on solid tumors.

Methods: In-vitro cytotoxicity was determined using HT-29 cells (human colon adenocarcinoma) plated in 96 well plates at a density of 5 X 10^4 cells/well. The cells were infected with Vac-PNP at multiplicities of infection (MOI) of 0.0 and 0.01. The cells were incubated overnight and then treated with fludarabine for five days at concentrations of 0, 50, 100, & 200 uM. Media containing the prodrug was changed daily. Cell viability was assessed with the WST-1 cell viability assay. In-vivo experiments were performed using two tumor models: HT-29 subcutaneous (SQ) tumors grown in athymic nude mice and MC-38 (murine colon carcinoma) SQ tumors grown in C57Bl6 mice. The nude mice were injected with 10^6 cells of the HT-29 line and were treated when the tumors reached 10 mm. 10^8 plaque forming units (pfu) of Vac-PNP were injected intraperitoneally (IP), and then on day #4 fludarabine was administered IP at 5 mg/day/animal in three divided doses per day for 10 days. The SQ tumors were serially measured. The C57Bl6 mice were similarly treated. 10^6 cells of the MC-38 line were injected SQ. When the tumors reached 10 mm, 10^8 pfu Vac-PNP was injected IP and fludarabine was administered IP at a dose of 5 mg/day/animal in three divided doses for 5 days. Similarly, the SQ tumors were serially measured.

Results: The in-vitro data demonstrated that the Vac-PNP/fludarabine combination is an effective suicide gene therapy system. No cytotoxicity was found when the cells were treated with up to 200 uM of fludarabine alone. However, Vac-PNP infection at an MOI of 0.01 caused marked cytotoxicity with only 50uM of fludarabine (40 % cell viability) compared with the cells that were treated with only Vac-PNP (80% cell viability) at day 2. In-vivo results corroborated the in-vitro data. In the athymic nude model, there was a statistical difference ($p < 0.0001$) in the tumor sizes between the animals treated with Vac-PNP(4273 +/-1164 mm^3) and Vac-PNP in combination with fludarabine (1492 +/- 299 mm^3) at day 42. In the C57Bl6 model, there was a statistical difference($p < 0.0001$) in the tumor sizes between the animals treated with Vac-PNP (4110 +/- 1415 mm^3) and Vac-PNP in combination with fludarabine(761 +/- 402 mm^3) at day 14. In both models vaccinia without prodrug significantly inhibited tumor growth compared to non-viral controls. Control animals and animals treated with only fludarabine had to be euthanized early in both experiments due to unchecked tumor growth.

Conclusions: Vaccinia virus expressing the purine nucleoside phosphorylase gene combined with fludarabine is an effective systemic in-vivo cancer therapy. These results support clinical investigation of this construct.

LB-35 "Blood Biopsy" for epithelial cancer cells based on circulating cancer cell tests. Ts'o, Paul O.P.; Lesko, Steve; Lauderdale, Vivian; Ohara, Karen; *Cell Works, Inc., Baltimore, MD 21224*

The importance of early detection of cancer recurrence after treatment of a primary tumor, early diagnosis of metastasis, and early information about the responsiveness to systemic chemical/hormonal therapies, are well recognized for management of cancer patients. By spiking cultured cancer cells from more than thirty cancer cell lines of Prostate, Breast, Colon, Gastric, Liver, Kidney cancers, etc. into 20 ml of human blood, Cell Works has established a universal procedure, the Circulating Cancer Cell (CCC) Test to detect circulating epithelial cancers. This procedure utilizes a double gradient sedimentation for the removal of most RBC and WBC as well as magnetic cell sorting for the additional removal of WBC before spreading the cancer cells onto a slide utilizing a cytospin apparatus. The fixed cells on the slide are then stained with various specific molecular probes, with selected fluorescent dyes attached. These cells are automatically scanned with an award winning spectroscopic microscope system, first in low magnification, where the fluorescent digital image is captured at a resolution of 0.2 μm using multiple excitation/

activation is detected in a majority of primary human breast tumor specimens examined compared to adjacent non-malignant tissues. Using Src and Janus kinase (JAK) selective inhibitors, we further demonstrate that functional c-Src and JAKs are required for constitutive activation of STAT3 in model human breast cancer cells. Treatment of these cells with Src or JAK selective inhibitors results in growth inhibition that is associated with abrogation of STAT3 DNA-binding activity, altered cell cycle progression and induction of programmed cell death. Consistent with the critical involvement of STAT3 in survival, a dominant-negative form of STAT3 induces programmed cell death in transfected breast cancer cells. Our findings suggest that constitutive activation of STAT3 by Src and/or JAKs prevents apoptosis of human breast cancer cells and thereby contributes to malignant progression. Preliminary analyses by microarray gene expression profiling based on Affymetrix DNA chips has identified a set of candidate genes that potentially participate in STAT3-dependent regulation of growth and survival of human breast cancer cells.

#3772 Stat3 Activation and Androgen-independent Progression of Prostate Cancer. Fernando DeMiguel, Beth Pflug, Joel Nelson, and Allen C. Gao. *University of Pittsburgh, Pittsburgh, PA.*

Almost all patients with advanced prostate cancer respond initially to androgen ablation and antiandrogen therapy. However, virtually every patient will relapse due to the growth of androgen-independent cancer cells. The molecular changes in prostate cancer cells leading to androgen-independent growth are incompletely understood. Stat3, a member of the Janus kinase (JAK)-Signal Transducers and Activators of Transcription (STAT) family, can act as an oncogene, playing a critical role in cell transformation and tumor progression. We have previously demonstrated that Stat3 is constitutively activated in prostate cancer cell lines, and blockade of Stat3 expression in human prostate cancer cells suppressed their proliferation in vitro and tumorigenicity in vivo. We now show that Stat3 activity is significantly increased in human primary prostate cancer tissues compared with normal prostate tissues. In addition, the level of Stat3 activity is increased in the androgen-independent cell lines derived from both androgen-dependent LNCaP and LAPC-4 human prostate cancer cell lines. Furthermore, activated Stat3 can partially rescue LNCaP cells from growth arrest induced by androgen deprivation in vitro. These results suggest that Stat3 activation play a critical role in androgen-independent progression of prostate cancer.

#3773 Mechanism of Constitutive Activation of NF-κB Transcription Factor in Human Prostate Carcinoma Cells. Alexander V. Gasparian, Yajuan Yao, Dariusz Kowalczyk, Thomas J. Slaga, and Irina V. Budunova. *AMC Cancer Research Center, Lakewood, CO.*

The transcription factor NF-κB regulates the expression of numerous genes involved in cancer cell growth, invasion, metastasis, angiogenesis, and resistance to chemotherapy. The key to NF-κB regulation is the inhibitory κB (IκB) proteins which retain NF-κB in an inactive form in the cytoplasm. In response to diverse stimuli, IκBs are rapidly phosphorylated, ubiquitinated and undergo degradation via 26S proteasome. The released NF-κB factors then translocate to the nucleus and activate κB-responsive genes. Several IκB kinases (IKKs) which trigger IκB degradation and NF-κB activation have been discovered recently, and are currently seen as a target of choice to specifically inhibit NF-κB activity. We studied the NF-κB expression and function in primary prostate epithelial cells, and five prostate carcinoma (PC) cell lines LNCaP, MDA PCa2b, DU145, PC3, and JCA1. Our results indicate that NF-κB is constitutively activated in human androgen-independent PC cell lines. In spite of strong difference in constitutive κB binding, Western blot analysis did not reveal any significant variance in the expression of p50, p65, IκB-α, IκB-β, and IκB kinases IKKα and IKKβ between primary prostate cells, androgen-dependent, and androgen-independent PC cells. However, we found that in all androgen-independent PC cells IκB-α was heavily phosphorylated and displayed a faster turnover than in androgen-dependent PC cells. Indeed, proteasomal inhibition by MG132 caused faster accumulation of phosphorylated IκB-α in androgen-independent cell lines. The blockage of IκB-α synthesis de novo by cycloheximide in combination with IKK blockage revealed faster degradation of phosphorylated IκB-α in androgen-independent cell lines. The latter result was confirmed by pulse chase analysis: the half-life time for IκB-α in LNCaP cells was almost twice longer than in DU145 and JCA1 cells. By in vitro kinase assay we demonstrated that IKK was constitutively active in androgen-independent PC cells. Conclusively, our data suggest that aberrant IKK activation leads to the constitutive activation of NF-κB "survival signaling" pathway in androgen-independent PC cells and may contribute to their growth advantage.

#3774 Progression of p53 Wild-Type Tumors in Vivo is Determined by FAS Inactivation. Heather L. Maecker, Zhong Yun, Holden T. Maecker, and Amato J. Giaccia. *BD Biosciences, Immunocytometry Systems, San Jose, CA, and Stanford University, Stanford, CA.*

We have previously reported that p53 selects for apoptotic resistance in vitro by inactivation of the Fas pathway through acetylation and repression of Fas gene expression. In the current study we tested whether or not a selective pressure for Fas inactivation existed in vivo during tumor progression of p53 wild-type tumors. To this end, MEF cells transformed with E1A/Ras either wild-type (+/+) or null (-/-) for p53 were injected s.c. into the flank of scid mice and tumor progression was followed over 30 days. While only one mouse injected with (+/+) early passage MEF cells, expressing high levels of surface Fas, developed a tumor (0.1 gm), all 5 mice injected with (-/-) MEFs grew tumors averaging 2 gm at day Furthermore, all 5 mice injected with late-passage (+/+) MEF cells, express low surface Fas levels, grew tumors reaching an average of 3.25 gm at day post-injection. To confirm that tumor take and progression in the late passa (+/+) MEF cells was due to their Fas expression, these cells were retrovira transfected with either Fas or DN-Fas constructs. These cells were injected ba into mice and their tumor progression is currently being studied.

#3775 Transcriptional Regulation of the p19ARF Tumor Suppres Gene by CTCF. Chen-Feng Qi, Elena Pugacheva, Joe Breen, David Lee, Dm Loukinov, Sergei Vatolin, Hanlim Moon, Thomas McCarty, Victor V. Lobanenk and Herbert C. Morse. *National Institute of Allergy and Infectious Diseas Bethesda, MD.*

CTCF is a multifunctional 11 zinc finger transcription factor originally identif as a repressor of *MYC* expression. Subsequent studies suggested that CTCF m also function as a transcriptional activator as demonstrated for the *APP* promo Expression of MYC is rapidly modulated at the transcriptional level when imn ture B cells are induced to undergo apoptosis by crosslinking surface immur globulin. Using the WEHI 231 cell line to model this phenomenon, we confirm that MYC is markedly upregulated by 2hr after induction, returning to basel levels by 4h but found that these changes cannot be ascribed to regulation CTCF. However, progressive increases in CTCF expression after crosslink were associated with greatly increased levels of p19/ARF. We showed by gel sh assays that the mouse $p19^{ARF}$ promoter contains a large CG-rich CTCF target s and identified the contact nucleotides by methylation interference. Cotransfect experiments with the wild type and CTCF site-mutated CTCF $p19^{ARF}$ promo showed that CTCF binding induces strong, specific transcriptional activation the $p19^{ARF}$ promoter. Using an IPTG-inducible CTCF system, we demonstrat induction of transcription from the endogenous $p19^{ARF}$ locus. These stud identify CTCF as prominent component of the p53-p19/ARF-MDM2 axis th modulates responses to normal and oncogene-induced proliferative stimuli.

#3776 p53 and Hypoxia Mediated Gene Repression. Ester M. Hammo and Amato Giaccia. *Stanford University, Stanford, CA.*

Hypoxia induced apoptosis signaled by p53 does not involve the transacti tion of known p53 target genes such as p21 and Bax. Preliminary data indicat that under hypoxia p53 acts as a transrepressor to induce apoptosis. The mec anism behind p53-mediated transrepression is unclear. In order to identify ger repressed by p53 in response to hypoxia we have used DNA microarrays. T cell systems have been considered, a p53 inducible form of H1299s and t HCT116 p53 null and positive cell lines. The data obtained has been clustered identify putative repressed genes. Repressed genes fell into at least 2 class Class 1, those that are induced by hypoxia but are induced less so in t presence of p53, and class 2, those that are repressed below basal levels in t presence of hypoxia and p53. These genes and the mechanisms of their repre sion have been examined in more detail.

#3777 Rvb1p/Rvb2p, Implicated in Cell Transformation by MYC and DNA Repair and Apoptosis, is an ATP Dependent Chromatin Remodeli Factor. Anindya Dutta, Zophonias Jonsson, Geeta Narlikar, Roy Auty, Nik Wagle, and Suman Dhar. *BWH, Harvard Medical School, Boston, MA, Harva Medical School, Boston, MA, and MGH, Harvard Medical School, Boston, MA*

Rvb1/TIP49a and Rvb2/TIP49b are two highly conserved proteins related to t helicase subset of the AAA family of ATPases that are associated with each oth M. Cole and co-workers reported that the Rvb proteins associate with t transforming domain of Myc and that a dominant negative mutant of Rv abrogates cell transformation by Myc. The mammalian Rvb proteins have be recently reported by Y. Nakatani and colleagues to be associated with the histo acetylase TIP60 and implicated in DNA repair and apoptosis. Here we report th the yeast Rvb1/2 proteins are individually essential for viability, require the A binding motifs for their normal function and are necessary for diverse process ranging from expression of galactose-responsive genes to maintenance of c cycle arrest in response to pheromones. Conditional mutants of RVB1 or RVE reveal that transcription of approximately 5% of yeast genes is either repress or activated by the Rvb proteins. Many inducible promoters are unable to ma tain the induced state after the inactivation of Rvb1 or Rvb2 indicating a role this factor in gene expression even after the establishment of a transcriptiona active initiation complex. The requirement of ATP binding sites, the positive negative effect on various yeast promoters and the regulation of a large numt of genes comparable to that regulated by the Swi/Snf chromatin remodeli complex led us to test whether Rvb1/Rvb2 are part of a new chromatin remo eling complex. Rvb complex purified from yeast possesses a chromatin stim lated ATPase activity and a potent chromatin remodeling activity on an array polynucleosomes in vitro. The Swi/Snf chromatin remodeling complex and Rvt Rvb2 proteins regulate different subsets of promoters, although individual p moters can be identified that are regulated by both complexes. Chromatin modeling has emerged as a key regulatory step in gene expression in eukaryo and our results identify a chromatin remodeling factor implicated in cell transf mation, DNA repair and apoptosis.

125 **p65-NFκB synergizes with Notch to activate transcription by triggering cytoplasmic translocation of the Nuclear Receptor Corepressor N-CoR.**

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Notch/RBPJκ and Nuclear Factor-κB (NFκB) complexes are key mediators in the progression of many cellular events by activating transcription of specific target genes. Independent observations have shown that activation of Notch dependent transcription generally correlates with inhibition of differentiation. In contrast, activated NFκB complexes are required for progression of differentiation in several systems. Although some interactions between both pathways have been observed, the physiological significance of their connection is unclear. We have now demonstrated that ectopic expression of p65-NFκB protein enhances up to three fold Notch-mediated activation of the Hes1 promoter.This effect does not require NF-κB transcriptional activity and it is independent of the previously described interaction between Notch and p50-NFκB. Furthermore, p65-NF-κB can trigger cytoplasmic translocation of the transcriptional corepressor N-CoR, thus abrogating N-CoR mediated repression of the Hes1 promoter. Thus, we conclude that p65-NFκB can regulate gene expression by a new mechanism that involves functional inhibition of N-CoR.

126 **Transgenic NF-kB Reporter Mice Reveal Constitutive NF-kB Activity That Is Required For Central Neuron Survival.**

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The function of NF-kB within the developing and mature central nervous system is controversial. To assess NF-kB activity in neurons *in vivo*, an NF-kB responsive ?-galactosidase minigene was used to generate transgenic mice. Constitutive NF-kB activity was present in transgenic immune organs and TNFα treatment of primary transgenic fibroblasts resulted in inducible ?-galactosidase activity that was inhibited by IkBαM overexpression. Developmental analysis revealed prominent NF-kB activity in the central nervous system that persisted to adulthood, especially within the neo-cortex, olfactory bulbs, amygdala, and hippocampus. In primary dissociated culture of transgenic E16 cortical cells, NF-kB activity was elevated by adenovirus-mediated overexpression of p65/RelA and reduced by adenovirus-mediated overexpression of the IkBαM super-repressor or dominant-negative NIK. Inhibition of NF-kB activity induced cortical neuron death whereas p65/RelA overexpression increased levels of Bcl-xL, IAP1 and IAP2 and conferred potent neuroprotection. Together, these data demonstrate an important role for NF-kB in the survival of CNS neurons.

127 **Selenium compounds inhibit IκB kinase and transcriptional factor NF-κB in prostate cancer cells.**

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Selenium (Se) compounds are potential chemopreventive agents for prostate cancer. There are several proposed mechanisms of their anticancer effect, including enhanced apoptosis of transformed cells. Because the transcription factor NF-κB is often constitutively activated in tumors and is a key anti-apoptotic factor in mammalian cells, we tested whether Se inhibited NF-κB activity in prostate cancer cells. Using sodium selenite and a novel synthetic compound methylseleninic acid (MSeA) that served as a precursor of the putative active monomethyl metabolite methylselenol, we found that both Se forms inhibited NF-κB DNA binding induced by TNF-α and LPS in DU145 and JCA1 cells. The kinetics of NF-κB inhibition was different with a quick and transient inhibition of κB binding by MSeA and a slower but more sustained inhibition by selenite. Both compounds also inhibited κB.Luciferase reporter activity in prostate cells. A key to NF-κB regulation is the inhibitory κB (IκB) proteins that in response to diverse stimuli are rapidly phosphorylated by IκB kinase (IKK) complex, ubiquitinated and undergo degradation, releasing NF-κB factor. We showed that selenite and MSeA inhibited IKK activation, IκB-α phosphorylation and degradation induced by TNF-α and LPS in prostate cells. The extent and persistence of NF-κB inhibition appeared to correlate with the Se effect on growth and survival of prostate cells. These results suggest that Se may target the NF-kB activation pathway to exert, at least in part, its cancer chemopreventive effect in prostate.

This work supported by funding from the DOD Prostate Cancer Research Program DAMD17-01-1-0015.

128 **Mechanism of melphalan-induced up-regulation of B7-1 gene expression**

Manjula Donepudi, Pradip Raychaudhuri, Margalit B. Mokyr, Department of Biochemistry and Molecular Biology, University of Illinois at Chicago, Chicago, IL, 60612.

We have previously shown that administration of a low dose of the widely-used anticancer drug melphalan (L-phenylalanine mustard, L-PAM) to MOPC-315 tumor bearing mice leads to the acquisition of $CD8^+$ T-cell-dependent tumor-eradicating immunity via a B7-dependent mechanism. In addition, we have shown that the chemotherapy is associated with rapid up-regulation of the surface expression of the costimulatory molecule B7-1 on both tumor cells and host antigen presenting cells. Here we show that *in vitro* exposure of tumor cells or host cells to L-PAM also leads to rapid up-regulation of B7-1 surface expression, and the induced B7-1 molecule is functional. In addition, we show that L-PAM-induced up-regulation of B7-1 surface expression requires *de novo* RNA synthesis and is associated with accumulation of B7-1 mRNA, indicating that the regulation is at the transcriptional level. The effect of L-PAM on B7-1 surface expression can be mimicked by exposing cells to oxidative stress, but not heat shock. Moreover, the antioxidant N-acetyl-L-cysteine (NAC) prevents the L-PAM-induced accumulation of B7-1 mRNA, suggesting that reactive oxygen species are involved in the transcriptional regulation of L-PAM-induced B7-1 expression. Although AP-1 and NF-κB are regarded as redox-sensitive transcription factors, and the promoter/enhancer region of the B7-1 gene contains an AP-1 and NF-κB binding site, exposure of cells to L-PAM leads to rapid and transient activation only of NF-κB, but not AP-1. Moreover, exposure of cells to a cell-permeable peptide that selectively inhibits NF-κB activation by blocking the activation of the IκB kinase complex inhibits the L-PAM-induced B7-1 mRNA accumulation, indicating that NF-κB activation is essential for the L-PAM-induced B7-1 gene expression. Taken together, these results indicate that L-PAM leads to the induction of B7-1 gene expression by activating NF-κB via a pathway that involves reactive oxygen species. These results have important implications for an additional immune potentiating mechanism of anticancer drugs in a clinical setting.

ERKs are inhibited in a parallel concentration-dependent fashion. These results are further supported by experiments where integrin $\alpha 2$ expression and activation of ERK-1 and ERK-2 were inhibited on treatment of cells with EGFr blocking mAb 528. The p38 MAPK inhibitor SB 203580 did not exhibit any significant inhibitory effect on integrin $\alpha 2$ expression or its biological functions. This demonstrated the selective participation of the MEK pathway as being essential for integrin $\alpha 2$ expression. Incubation of cells with a selective inhibitor of PI3K, LY294009, did not affect integrin $\alpha 2$ expression. Thus, basal control of cell adhesion and cell motility on collagen IV appear to be under the autocrine TGFα control leading to constitutively activated MEK and ERK. Thus, ERKs may play an important role in cell adhesion and integrin $\alpha 2$ expression which in turn may control cell survival mechanism(s) in HCT116 cells. Constitutive enhancement of motility by autocrine growth factors in malignant cells may be an important contributory factor to invasive and metastatic properties exhibited by HCT116 cells.

#1807 Glial cell-induced endothelial morphogenesis is inhibited by interfering with ERK signaling. Nirmala Chandrasekar, Sanjeeva Mohanam, Sajani S. Lakka, Deeba Yunus, Dzung H. Dinh, William C. Olivero, and Jasti S. Rao. *University of Illinois College of Medicine, Peoria, IL.*

Because tumor vasculature provides the infrastructure by which malignant tissue can be nourished, angiogenesis may be an effective target for treatment of cancer. We previously demonstrated that SNB19 glioblastoma cells stimulate bovine retinal endothelial cells to form a capillary-like network, a process that involves proteolysis, adhesion, and migration of the endothelial cells. We also found that matrix metalloproteinase-9 (MMP-9) expression is critical for endothelial morphogenesis. MMP-9 expression seems to be regulated by extracellular signal-regulated kinase-1 (ERK-1) in glioblastoma cells. In the present study, we attempted to determine whether interfering with the activation of this MAPK can repress MMP-9 synthesis and inhibit capillary formation. The kinetic profiles of ERK activity in SNB19-bovine retinal endothelial cell cocultures suggested that prolonged activation of ERK underlies the stimulation of endothelial-cell morphogenesis and MMP-9 levels. We found that inhibition of the ERK1/2 pathway with PD98059, a specific inhibitor of MEK1, abrogated the glial cell-mediated capillary formation by endothelial cells and reduced the levels of MMP-9 in the coculture. Also, the abrogation of MAPK signaling by a dominant-negative ERK-1 mutant strikingly inhibited capillary-network formation as well as MMP-9 levels. Our findings demonstrate that ERK inhibition not only reduces MMP-9 activity but also inhibits the adhesion and migration of endothelial cells, thereby inhibiting capillary formation. Interfering with ERK signaling seems to be a viable pathway for targeting angiogenesis.

#1808 Prostate carcinoma cells secrete a soluble factor(s) responsible for NF-κB activation. Alexander Gasparian, Alexander Yemelyanov, Ya Juan Yao, Dariusz Kowalczyk, and Irina Budunova. *AMC Cancer Research Centre, Denver, CO.*

We found recently that NF-κB is constitutively activated in human androgen-independent prostate carcinoma cell lines DU145, PC3, and JCA1 but not in normal prostate epithelial cells and androgen-dependent PC cell line LNCaP. It was shown that prostate carcinoma cells produce and secrete some cytokines and growth factors that are potential NF-κB activators. It is also known that some of these cytokines and growth factors are themselves regulated by NF-κB at the transcriptional level. We hypothesized that increased NF-κB activity in androgen-independent PC cells may be mediated by such factor(s) through an autocrine/paracrine loop. To test this hypothesis we incubated LNCaP cells with the conditioned medium of PC3 cells. Conditioned medium taken from PC3 cells induced IκBα phosphorylation as well as κB-binding in LNCaP cells. It also increased the expression of endogenous κB-responsive genes such as IκB-α The conditioned medium of DU145 and JCA1 cells did not affect NF-κB activity in LNCaP cells. Boiling and multiple freezing/thawing of PC3 conditioned media eliminated the effect of the medium on κB binding. This suggests that the secreted NF-κB inducer is a proteinaceous factor. The nature of the factor/factors that induce NF-kB is under study. Supported by DOD Prostate Cancer Research Program DAMD17-01-1-0015.

#1809 Rap1, a ras-like protein localizes to the nucleus in head and neck squamous cell carcinoma (HNSCC). Nisha J. D'Silva, Raj Mitra, Bradley Henson, Zhaocheng Zhang, David Kurnit, and Thomas Carey. *University of Michigan, Ann Arbor, MI.*

Over 100 small GTP-binding proteins (smgs) are expressed in eukaryotes. These proteins play a critical role in cellular functions including growth and differentiation, gene expression and cytoskeletal reorganization. Of these proteins, Ran/TC4 is the only smg that has been identified in the nucleus where it is thought to play a role in growth and proliferation by regulating nucleocytoplasmic transport. In this report we describe a novel localization of rap1, another smg, in the nucleus of HNSCC cells. Rap1 exists in two isoforms, rap1A and rap1B that are encoded by genes on chromosomes 1 and 12, respectively. Methods: Expression of rap1 in human HNSCC cell lines or in 3T3 fibroblasts transfected with wild type rap1A or rap1B was examined by immunohistochemistry and immunoblot analysis of whole cell lysates and nuclear and cytoplasmic subcellular fractions. Rap1 expression in tissue sections from 15 different human intraoral squamous cell carcinomas was evaluated by immunohistochemistry. Furthermore, rap1A and rap1B isoforms were identified in HNSCC by quantitative real time reverse transcriptase polymerase chain reaction (Q-RT-PCR) and blot analysis. Results: Variable rap1 expression was detected by imr analysis of the whole cell lysate of a selection of 10 HNSCC cell lines. most strongly expressed in whole cell lysates of UM-SCC-17B foll UM-SCC-81B and shows a low level of expression in OSCC3, a poorly tiated oral cancer cell line. Immunoblot analysis of nuclear and cyt fractions and immunohistochemistry on UM-SCC-22B and UM-SCC- representative cell lines showed that rap1 is strongly expressed in the whereas weak cytoplasmic staining is detected only in UM-SCC-17B. B isoforms were identified in all HNSCC cell lines by Northern blot anal Q-RT-PCR. All intraoral SCC tissues sections examined strongly express Strong cytoplasmic expression of rap1 was noted in large differentiated n keratinocytes whereas nuclear and cytoplasmic localization were ide cells that were morphologically less differentiated. 3T3 fibroblasts tra with wild type rap1A or rap1B exhibited nuclear and cytoplasmic locali both these rap1 isoforms whereas endogenous rap1 was weakly detect empty vector control. Conclusions: The nuclear localization of rap1 is finding of this study and represents the first report of nuclear localization other than ran. By analogy with the ran subfamily proteins, rap1 may growth and proliferation by regulating nucleocytoplasmic transport.

#1810 pp32 gene family expression in cancer and the balance l proliferation and differentiation. Jonathan Robert Brody, Shrihari Kadk erick Racke, Michael Trush, and Gary R. Pasternack. *The Johns Hopkin sity School of Medicine, Baltimore, MD, and The Johns Hopkins Universit of Public Health, Baltimore, MD.*

pp32 is a critical component of a complex that regulates immedi mRNA stability, histone acetylase, and a caspase-independent apoptc way. At least three other highly conserved pp32 gene family member coded by distinct genes located on different chromosomes, however th cancer and in normal development is unclear. Multiple members of the p family may be expressed in human breast, prostate, and other cancers, corresponding normal tissues that express principally pp32. Functionally nuclear phosphoprotein, acts as a tumor suppressor in contrast to pp pp32r2, which are oncogenic in some systems. To date, no mutations h found in pp32 mRNA sequences from normal or neoplastic tissues. Expr pp32 message and protein is highly regulated in a differentiation-d fashion in the myeloid leukemic cell lines ML-1 and K562, and in a adenocarcinoma cell line, TSUPr-1. TPA-induced differentiation of the leukemia cells caused a marked decrease in pp32 expression as th terminally differentiated and stopped proliferating, correlating with expr myeloid differentiation markers including NADPH oxidase and CD41. In to pp32, pp32r1 and pp32r2 expression did not change significantly up entiation induction. TSUPr-1 cells express pp32>>pp32r2>pp32r1 transfection of TSUPr-1 with pp32 sense substantially reduced the n clones obtained in comparison to control, whereas stable transfectior anti-sense pp32 cDNA construct, that abolishes all pp32 family membe sion, yielded markedly an increased number of clones, consistent with t suppressor role of pp32. These results confirm our previous findings t overexpression reduces cancer cell proliferation. In contrast, pp32 ge depletion provides cancer cells with a proliferative advantage. Modulati balance of expression of pp32 gene family members may alter the between proliferative potential and the ability to differentiate

#1811 A novel approach to isolate cell cycle regulators: Functio acterization of LETM-1 which causes G0/G1 cell cycle arrest. Y Hitoshi, Denise Pearsall, Jeff Quast, Susan Demo, Michel Janicot, Jorg Walter Luyten, Walter Woulters, Donald Payan, James Lorens, Susan M and Mark Bennett. *Rigel Inc, South San Francisco, CA, and Janssen Insitute, Beerse, Belgium.*

In recent years, there have been major developments in understandi cell cycle. Normal cell proliferation is tightly regulated by activation and tion of a series of proteins that constitute the cell cycle machinery. Expre activity of components of the cell cycle can be altered during develop variety of human diseases such as cancer, cardiovascular disease, and where aberrant proliferation contributes to pathology of the illness. regulation is highly conserved among all eukaryotic cells and genetic s various organisms such as yeast, worms, and flies, have yielded critical r of this cellular process. However there is a need to establish screens cells to better understand how disruption of cell cycle regulation causes Using a retroviral cDNA/peptide library-based functional screen with a latable gene expression system, we have identified several molecules ca cycle arrest in human cells. Here we report the identification and characterization of a surface molecule, leucine zipper-EF-hand contain membrane protein 1 (LETM-1), through a retroviral cDNA library-based screen. LETM-1 was originally identified as the gene flanking the critical the Wolf-Hirschhorn syndrome (WHS), a chromosomal disorder charact a 4p monosomy that results in certain facial dysmorphisms and ne manifestations. Our results demonstrate that LETM-1 overexpressio potent G0/G1 arrest in a p53-independent and Rb-dependent manner,

microarray analysis of the gene expression profiles revealed a panel of genes, involved in cell cycle regulation and apoptosis, regulated by IL-4 in a STAT6 dependent manner.

#2727 Inhibition of constitutive STAT3 activity sensitizes resistant non-Hodgkin's lymphoma and multiple myeloma to chemotherapeutic drug-mediated apoptosis. Steve Alas and Benjamin Bonavida. *City of Hope, Duarte, CA, and University of California, Los Angeles, Los Angeles, CA.*

Hematopoietic malignancies have been shown to depend on cytokine growth factor autocrine/paracrine loops for growth and differentiation. This results in the constitutive activation of cytokine-mediated transcription factors like STAT3 in non-Hodgkin's lymphoma (NHL) and multiple myeloma (MM). Recent evidence demonstrates that cytokines also contribute to a drug-resistant phenotype in many tumor cell types. We hypothesized that inhibitors of STAT3 would sensitize drug-resistant and endogenous cytokine-dependent NHL and MM tumor cells to the cytotoxic effects of chemotherapeutic drugs. We examined an AIDS-related NHL (ARL) cell line, 2F7, known to be dependent on IL-10 for survival and an MM cell line, U266, known to be dependent on IL-6 for survival. IL-10 and IL-6 signal the cells through the activation of JAK1 and JAK2, respectively. Thus, we investigated the effect of two chemical STAT3 inhibitors, namely, piceatannol (JAK1/STAT3 inhibitor) and tyrphostin AG490 (JAK2/STAT3 inhibitor) on the tumor cells for sensitization to therapeutic drugs. We demonstrate by phosphoprotein immunoblotting analysis and electrophoretic mobility shift analysis that piceatannol and AG490 inhibit the constitutive activity of STAT3 in 2F7 and U266, respectively. Furthermore, piceatannol and AG490 sensitize 2F7 and U266 cells, respectively, to apoptosis by a range of therapeutic drugs including cisplatin, fludarabine, adriamycin and vinblastine. The specificity of the inhibitors was corroborated in experiments showing that piceatannol had no effect on U266 and, likewise, AG490 has no effect on 2F7. The sensitization observed by these inhibitors correlated with the inhibition of Bcl-2 expression in 2F7 and Bcl-xL expression in U266. Altogether, these results demonstrate that STAT3 inhibitors are a novel class of chemotherapeutic sensitizing agents capable of reversing the drug-resistant phenotype of cytokine-dependent tumor cells.

#2728 STAT3 activation accompanies keratinocyte differentiation. Rong Wu, Shishinn Sun, and Bettie M. Steinberg. *Department of Otolaryngology, North Shore-Long Island Jewish Research Institute, Long Island Jewish Medical Center, New Hyde Park, NY.*

STAT3, a member of the signal transducers and activators of transcription (STAT) family of transcription factors, has been shown to play a key role in differentiation of haematopoietic cells. Laryngeal papillomas, benign tumors induced by human papillomavirus (HPV) 6/11, have alterations in a number of signaling pathways. They overexpress the EGF receptor, display enhanced MAP kinase and PI-3-kinase activity, and show reduced activated Akt due to overexpression of the tumor suppressor PTEN. We have now found that they also have reduced levels of phosphorylated STAT3. Papillomas are characterized by abnormal terminal differentiation, compared to uninfected laryngeal epithelium, suggesting that STAT3 may play a role in differentiation of stratified squamous epithelium. To examine the potential role for STAT3 in regulating epithelial differentiation, we have established a suspension culturing system that induces differentiation of mucosal epithelium. Cultured epithelial cells were suspended in serum-free medium in the presence of EGF and insulin, under conditions where cell-cell contact was enhanced but cell-matrix contact was blocked. Effects on differentiation and STAT3 activation were determined 48 hours later by Western blot and immunofluorescence staining. Activation of STAT3 was enhanced, accompanied by increased expression of the specific differentiation marker keratin 13. We propose that activation of STAT3 is required for differentiation of stratified squamous epithelium, as it is for haematopoietic cells. Experiments are in progress, using constitutively active and dominant negative constructs of STAT3, to test this hypothesis.

#2729 Role of prolonged Elk-1 phosphorylation in growth inhibition of hepatoma cells by a new K vitamin analog. Takahito Adachi and Brian I. Carr. *University of Pittsburgh, Pittsburgh, PA.*

Compound 5 (Cpd 5) or 2-(2-mercaptoethanol)-3-methyl-1,4-naphthoquinone, a synthetic K vitamin analog inhibits cell growth via inhibition of protein-tyrosine phosphatases, resulting in prolonged EGF and ERK phosphorylation (Proc. AACR 42:301, 2001). We have now examined likely consequences of these actions. We found that Cpd 5 induced a strong and sustained phosphorylation of Elk-1, a nuclear target of phospho-ERK, in contrast to the weak and transient effect of EGF. This induction was concentration-dependent and lasted for several hours, unlike the transient effect of EGF. Elk-1 phosphorylation was inhibited by U0126, a MEK inhibitor, which also antagonized the growth inhibitory effects of Cpd 5. Furthermore, dephosphorylation by cell lysates of exogenous phospho-Elk-1 protein was also inhibited by pre-treatment with Cpd 5. However, DNA binding of Elk-1 and the downstream transcription factor AP1, was prevented by Cpd 5, while EGF increased it. We also examined the AP-1 components c-Jun, JunB and JunD. Cpd 5 induced prolonged phosphorylation of JNK, an upstream MAPK of c-Jun, but c-Jun phosphorylation was transient. JunD was phosphorylated gradually by Cpd 5 treatment, while JunB was not induced. These results show that Cpd 5 induced prolonged phosphorylation of Elk-1 via its inhibitory effects on Elk-1-specific phosphatase(s) and the suppression of phospho-Elk-1 and AP-1 activities are probably involved in Cpd 5 mediated growth inhibition.

#2730 The promyelocytic leukemia protein PML is a functional repressor of NF−κ B. Wen-Shu Wu, Walter N. Hittelman, and Kun-Sang Chang. *UT MD Anderson Cancer Center, Houston, TX.*

The chromosomal translocation breakpoint t(15;17), a consistent cytogenetic feature of acute promyelocytic leukemia (APL) disrupts the growth suppressor gene *PML*. Recent studies demonstrated that PML plays an essential role in multiple pathways of apoptosis and cell cycle regulation. Our study here demonstrated that PML is a transcriptional repressor of NF-κB. Our results showed that cotransfection of PML expression plasmid and NF-κB-dependent reporter dramatically repressed transactivation of NF-κB induced by TNF-α. PML has little effect on degradation of I-κB. In a series of cotransfection experiments using RelA (p65), PML expression plasmid and NF-κB reporter construct, PML significantly repressed the transcriptional activity of RelA in a dose-dependent manner. To further elucidate how PML inhibits NF-κB function, EMSA was performed using *in vitro* translated PML. We found that PML inhibits RelA from binding to its consensus enhancer sequence. This study suggests that PML is a functional repressor of NF-κB. We next investigate whether PML and NF-κB are associated *in vivo*. This study showed that PML Co-IP RelA and that the two proteins are colocalized *in vivo* in the cotransfection experiment. Result further showed that the C-terminal of PML is essential for such association. To study the *in vivo* association between PML and NF-κB, endogenous co-IP and double color immunofluorescent staining were performed using U2OS and Siha cell lines. This study demonstrated that PML is associated with NF-κB at the endogenous level. Increased cancer cells survival attributed frequently to the activation of NF-κB. Our study here supports a novel mechanism of PML induced cell death through inhibition of the NF-κB survival pathway.

#2731 Role of IKKe in NF-kappaB activation in prostate carcinoma cells. Ya Juan Yao, Dariusz M. Kowalczyk, and Irina V. Budunova. *AMC Cancer Research Center, Denver, CO.*

Activation of NF-κB transcription factor by different inducers employs a common pathway. IκB inhibitory proteins, which retain NF-κB in the cytosol, are rapidly phosphorylated by IκB kinases (IKKs), ubiquitinated and undergo degradation. It became clear that NF-κB activation requires multiple distinct IKKs. IKKε (previously termed IKKi) is the only inducible IKK whose expression level is regulated by cytokines and possibly by NF-kB itself. This suggests that IKKε is involved in a positive autocrine loop of NF-κB activation. NF-κB transcription factor plays an important role in tumor development and is often constitutively activated in different tumor cells including androgen-independent prostate carcinoma (PC) cell lines (Gasparian, Budunova et al., 2001). The goal of this project was to study the role of IKKε in constitutive and inducible NF-κB activation in PC cells. Northern blot analysis revealed that IKKε is expressed in DU145 and PC3 cells, which have high constitutive levels of NF-kB activity, but not in primary normal prostate epithelial cells and in PC cell lines LNCaP and MDA PCa 2b which have low levels of NF-κB activity. IKKε transcription can be induced in PC cells by TNF-α treatment. The blockage of IKKε function by stabile transfection of PC3 cells with IKKε dominant negative (d.n.) mutant K38A (Peters et al., 2000) resulted in the decrease of constitutive expression of endogenous κB-responsive genes IκB-α and IL6. The IKKε d.n. mutant also significantly affected NF-κB activation by selective NF-κB inducers such as IL-1 and TPA. On the other hand, IKKε d.n. mutant did not affect activation of NF-κB-binding by TNF-α even though the degradation of IkB-α in IKKε d.n.-transfected cells was significantly blocked in comparison to parental and empty vector-transfected PC3 cells. These data indicate that IKKε is necessary for NF-κB induction by selected NF-κB stimuli and possibly plays a significant role in constitutive activation of NF-κB factor in androgen-independent PC cells. Supported by DOD Prostate Cancer Research Program DAMD17-01-1-0015.

#2732 NF-κB is probably not involved in cyclooxygenase-2 overexpression in murine colon cancer cells. Alexander Konson, Abed N. Azab, Gilad Rimon, Abraham Danon, and Riad Agbaria. *Ben-Gurion University of the Negev, Beer Sheeva, Israel.*

Cyclooxygenase-2 (COX-2), an inducible isoform of cyclooxygenase, is a rate-limiting enzyme for the biosynthesis of prostaglandins. COX-2 is consistently overexpressed in diverse tumors and has been implicated in tumor promotion and resistance to chemotherapy and radiation. Regulation of COX-2 expression is complicated and appears to involve diverse mechanisms in different cell types under various conditions. Several kinases and transcription factors are involved in the signal transduction pathways regulating COX-2 expression including: Ras, MAP kinase, PI3K/Akt and NF-κB. Many studies demonstrated the involvement of NF-κB in COX-2 expression in cells stimulated to express COX-2. However, the exact mechanism, which leads to COX-2 overexpression in tumor cells, such as colon cancer, is not known. The aim of the present study is to explore whether NF-κB is involved in COX-2 expression in unstimulated murine colon cancer cells (MC-38). Western blot analysis showed that COX-2 is expressed in MC-38 cells and they secrete high levels of prostaglandin E2 (PGE2). Inhibition of PGE2 secretion by NS398, a selective COX-2 inhibitor, showed that COX-2 is involved in prostaglandin synthesis in these cells. Moreover, cycloheximide, de novo

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HIGH SENSITIVITY OF PROSTATE CARCINOMA CELL LINES TO NF-κB INDUCTION.

Alexander V. Gasparaian, Ya Juan Yao, Thomas J. Slaga, Irina V Budunova
Institute of Carcinogenesis, N. Blokhin Cancer Research Center, Moscow, Russia, AMC Cancer Research Center, Denver CO and AMC CANCER RESEARCH CENTER, Lakewood CO

One of the central mechanisms protecting cells from apoptotic death is mediated by NF-κB transcriptional factors that control function of numerous cell survival genes. Our recent data and data by others showed that NF-κB is constitutively activated in androgen-independent prostate carcinoma (PC) cells and prostate tumors, and that NF-κB activation promotes PC cells resistance to apoptosis induced by chemo-therapeutical compounds. The results of our experiments indicated that androgen-independent PC cells maintain the high level of NF-κB basal activity by employment of the mechanism similar to that for NF-κB activation by inducers such as cytokines. This includes constitutive IKK activation, phosphorylation and fast turnover of IκBα inhibitor in androgen-independent PC cells. To find whether the high basal level of NF-κB activity in PC cells affects further NF-κB induction, we analyzed the sensitivity of normal prostate epithelial cells and PC cell lines to the standard NF-κB inducers such as TNF-α, TPA and LPS. The results of our experiments showed that in contrast to other tumor cell types with constitutively activated NF-κB, PC cells independently on the basal level of NF-κB, are highly sensitive to NF-κB activation. The lack of response of LNCaP cells to LPS and DU145 cells to TPA rather reflects the cell-specific changes in the upstream signaling than function of NF-κB transcription factor. Supported by DOD Prostate Cancer Research Program DAMD17-01-1-0015.

NF-κB induction in PC cells with low and high basal NF-κB activity.

Cell line	Basal κB binding	TNFα	TPA	LPS
PrEC*	Low	+++	+++	+++
LNCaP	Low	+++	++	-
MDA PCa 2b	Low	+++	+++	+++
DU145	Low	+++	-	+++
PC3	High	++	+++	++
PrEC* - normal prostate epithelial cells				

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showed that the kinase activity of Fer (Fer dominant negative) was determinant for STAT3 phosphorylation in PC-3 cells but not for the formation of Fer/STAT3 signaling complexes. The significance of these in vitro findings was demonstrated by immunohistochemistry in human prostate specimens from patients with advanced prostate cancer where Fer and STAT3 were expressed in the nucleus of the same tumour cell populations. Altogether these findings show for the first time the implication of the Fer kinase in the modulation of prostate cancer cell growth by IL-6 and its major role in the activation of STAT3 up to the nucleus. We propose a novel role for Fer in maintaining elevated levels of pY-STAT3 for binding to the promoter region of IL-6 dependent target genes. Supported by the Cancer Research Society, Inc.

#4298 Role of p42/44 mitogen activated protein kinase signal transduction in PC3 cells, a line of hormone independent prostate cancer cells. Hari K. Koul, Lakshmi Chaturvedi, Mei Yi Haung, Akshay Bhandari, Mani Menon, Ira Wollner, George Divine, Raymond Demers, Anil Wali, and Roger J. Davis. *Henry Ford Health Sciences Center, Detroit, MI, Karmanos Cancer Institute, Detroit, MI, and Howard Hughes Medical Institute and Program in Molecular Medicine, Worcester, MA.*

Prostate cancer is the second leading cause of cancer related deaths in males. Over the last decade, significant progress has been made in various treatment options for localized prostate cancer including surgical refinements as well as establishment of Hormone and Radiation therapy. However, to date no satisfactory treatment options are available for hormone resistant prostate cancer. The present studies evaluated the role of p42/44 Mitogen activated Protein Kinases (p42/p44 MAP kinase) signal transduction pathway in growth, viability and clonogenic activity of hormone-independent prostate cancer cells. For these studies, log phase cultures of PC3 in DMEM/F12 medium supplemented with FCS (10%) and antibiotics were grown in multi-well plates in the presence or the absence of PD 098059, a specific inhibitor of MEK (the upstream activator of p42/p44 MAPK) for 2 to 9 days. At the end of pre-determined time points, the rate of DNA synthesis was evaluated by 3H-thymidine incorporation; Kinetics of cell growth were measured by measuring cell numbers at given intervals by crystal violet staining; MAP kinase activity was measured by Western blot analysis using specific antibodies; Low melt agarose cultures were used in clonogenic assays. PC3 cells achieved linear growth pattern up to 6 days in culture and the growth rate tapered thereafter. The maximum rate of DNA synthesis was observed on day 5, thereafter rate of DNA synthesis tapered. These data suggest that maximum number of cells were in S-phase of cell cycle on day 5. Exposure of the cells to PD 098059(25 or 50 mM) had a modest effect on DNA synthesis in PC3 cells. Maximum effect of PD 098058 was observed on day 2 of exposure (~48% inhibition of the DNA synthesis with 50 mM, PD). The inhibition tapered by day 5 of exposure (to ~28% inhibition of the DNA synthesis with 50 mM, PD). PD 098059 decreased cell growth modestly (by 7-22%). Maximum inhibition of the cell growth was observed on day 5 of exposure (~22 % decrease in cell number with 50 mM, PD. The effects of PD 098059 on DNA synthesis and cell growth demonstrate that p42/p44 MAP kinase pathway plays only a minor role in the growth and viability of androgen-independent prostate cancer cells (PC3 cells). However, inhibition of ERK activity in PC3 cells had a profound effect (PD inhibited colony formation by over 80%) on clonogenic activity. These data suggest critical role for signal transduction via ERK pathway in synthesis and secretion of extracellular matrix components and thereby in tumor metastasis. Taken together these data demonstrate specific role for p42/p44 MAP kinase pathway in clonogenic activity of PC3 cells, and suggest critical role for this MAP kinase pathway in metastasis of hormone independent prostate cancer.

#4299 Interleukin-17 cytokines and novel receptor-like protein in prostate cancer. Dominik R. Haudenschild, Timothy A. Moseley, and A. Hari Reddi. *University of California at Davis Medical Center, Sacramento, CA.*

Chronic inflammation of the prostate may be a contributing factor in the development of prostate cancer. Members of the newly identified interleukin-17 cytokine family are produced by a variety of tissues including the prostate, although the founding member, IL-17, is expressed only by T-cells and B-cells. The functions of IL-17 cytokines in the prostate are not known. However, in other tissues IL-17s induce the expression of IL-6, IL-8 and iNOS, synergistically increase the effects of pro-inflammatory cytokines including IL-1 beta, IFN-gamma and TNF-alpha, and enhance the recruitment of neutrophils and macrophages. We therefore investigate the potential role of IL-17s in prostate and prostate cancer. We document the expression of IL-17 cytokines in the prostate, and describe the cloning and characterization of a novel type I single-pass transmembrane protein with homology to the IL-17 receptor (named IL-17 Receptor-Like, IL-17RL). High mRNA levels of IL-17RL were detected in prostate, cartilage, kidney, liver, heart, and muscle by northern blot. At least 14 RNA splice variants were found, transcribed from 19 exons on human chromosome 3. Alternative splicing was predicted to introduce premature stop codons, which often occur before the transmembrane domain. Translated proteins are predicted to range from 186 to 720 amino acids in length, and can be classified as either transmembrane or secreted proteins. The transmembrane proteins have cytoplasmic tyrosines, serines and threonines and thus the potential for signal transduction. The soluble secreted proteins lack the transmembrane and intracellular domains, and may function as soluble 'decoy receptors', retaining the ligand binding domain but acting as antagonists to cytokine signaling. Differential exon usage was found in different tissues by quantitative RT-PCR, raising the possibility that alternative splicing may regulate the activity of this pathway. Using antibodies directed to the cytoplasmic and the extracellular domains of IL-17RL, we investigated its expression in human prostate biopsies. Proteins corresponding in size to the soluble and the transmembrane isoforms are present in homogenized prostate biopsy, by western blot analysis. IL-17RL is distributed in both the epithelial and the stromal components of normal prostate, shown by immunohistochemistry. Moreover, there is evidence that the expression and distribution of IL-17RL may be altered in higher grades of carcinoma, and in areas of inflammation.

#R4300 IKKi is a component of the positive feedback loop involved in the constitutive activation of NF-κB in prostate carcinoma cells. Alexander Yemelyanov, Ya Juan Yao, and Irina V. Budunova. *AMC Cancer Research Center, Denver, CO.*

Our recent data and data by others indicate that NF-kappaB is constitutively activated in androgen-independent prostate carcinoma (PC) cells and prostate tumors, and that NF-kappaB activation promotes PC cell tumorigenicity, invasiveness and resistance to apoptosis. The important step in NF-kappaB activation is the phosphorylation of IkappaB inhibitor proteins by IKK kinases: IKKa, IKKb and IKK-related inducible kinase IKKi. IKKi is the only IKK whose activity is regulated by its expression. We found that IKKa and IKKb were uniformly expressed in primary prostate cells and PC cell lines. On contrast, IKKi was strongly expressed only in androgen-independent PC cells (PC3 and DU145) with high level of constitutively active NF-kappaB but not in androgen-dependent PC cell lines (LNCaP and MDA PCa 2b) and primary prostate epithelial cells. Immunostaining also revealed that IKKi was expressed in human prostate carcinomas. Treatment of PC cells with NF-kappaB inducers such as IL-1 and TNF-alpha resulted in a rapid induction of IKKi. Transient transfection of different PC cell lines with IKKi w.t. resulted in activation of kB.Luciferase reporter, whereas IKKi dominant negative (d.n.) mutant K38A suppressed basal NF-kappaB activity in PC cells. These data provide experimental evidence that IKKi could be involved in the regulation of NF-kappaB activity in PC cells through a positive feedback loop. Supported by DOD Prostate Cancer Research Program DAMD 17-01-1-0015 and University of Colorado prostate SPORE developmental program.

#R4301 Bone morphogenetic protein signaling in prostate cancer cell lines. Kristen D. Brubaker, Eva Corey, Lisha G. Brown, and Robert L. Vessella. *University of Washington, Seattle, WA.*

Bone morphogenetic proteins (BMPs), a subfamily of the transforming growth factor-ϵ^2 (TGF-ϵ^2) superfamily, regulate many developmental processes and are involved in bone formation. BMPs signal through a complex of type I and II receptors, which initiates a cascade of events that regulates these processes. Prostate cancer cells were found to express BMPs, which might contribute to the development of the osteoblastic lesions associated with CaP. Additionally, CaP cells were found to express BMP receptors (BMPRs) suggesting a possibility of direct effects of BMP on CaP cells. In this study we evaluated the

109 **IκB kinase independent IκBα degradation pathway: Functional NFκB activity and implications for cancer therapy**

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Antiapoptotic activity of NFκB in tumors contributes to acquisition of resistance to chemotherapy. Degradation of IκB is a seminal step in activation of NFκB. The IκB kinases, IKK1 and IKK2 have been implicated in both IκB degradation and subsequent modifications of NFκB. Using mouse embryo fibroblasts (MEFs) devoid of both IKK1 and 2 genes (IKK1/2$^{-/-}$), we document a novel IκB degradation mechanism. We show that this degradation induced by chemotherapeutic agent, doxorubicin (DoxR), does not require the classical serine 32 and 36 phosphorylation or the PEST domain of IκBα. Degradation of IκBα is partially blocked by PI3kinase inhibitor LY294002 and is mediated by the proteasome. Free NFκB generated by DoxR induced IκB degradation in IKK1/2$^{-/-}$ cells is able to activate chromatin based NFκB reporter gene and expression of the endogenous target gene, IκBα. These results also imply that modification of NFκB by IKK1 or IKK2 either prior or subsequent to its release from IκB is not essential for NFκB mediated gene expression at least in response to DNA damage. Additionally, DoxR induced cell death in IKK1/2$^{-/-}$ MEFs is enhanced by simultaneous inhibition of NFκB activation by blocking the proteasome activity. These results reveal an additional pathway of activating NFκB during the course of anti-cancer therapy and provide a mechanistic basis for the observation that proteasome inhibitors could be used as adjuvants in chemotherapy.

110 **NF-κB control of B cell development**

E Claudio, K Brown, S Park, H-S Wang and U Siebenlist, LIR, NIAID, NIH, Bethesda, MD, 20892.

NF-κB is critical for immune responses to pathogens. This family of transcription factors is also intimately involved in the development of hematopoietic lineages. We have investigated the role of NF-κB in B cell development aided by analysis of mice lacking the NF-κB1 and NF-κB2 proteins p105/p50 and p100/p52, respectively. As described previously, these double knockout mice have a B cell-intrinsic defect that blocks their progression past the transitional-1 stage in spleen, preventing the generation of mature B2 cells and marginal zone B cells. NF-κB activation at the transitional-1 stage is mediated at least partly via BAFF receptor-induced processing of p100/NF-κB2 to p52. BAFF is a member of the TNF family and it promotes survival of Transitional-1 B cells (Claudio et al. (2002), Nat. Immunol.3:958). We now demonstrate that the block in developmental progression of NF-κB1/2-deficient transitional-1 B cells is largely overcome with over-expression of the anti-apoptotic protein Bcl-2, although this does not fully restore B cell maturation. This confirms a survival role for NF-κB at this stage. We have also identified two more stages of B cell development that require the NF-κB1/2 proteins. The generation of small preB cells in the bone marrow is partially impaired in the absence of these NF-κB factors, a defect which is dramatically revealed when mutant cells are made to compete with wild-type cells in adoptive transfers.
Finally, the B1 B cell population, normally abundant in the peritoneum, is absent in mice lacking NF-kB1 and NF-kB2. Given the uncertain origin of B1 B cells, it remains to be shown whether their absence here is related to the bone marrow defect.

111 **Human Cytomegalovirus Infection of ARPE-19 Cells Downregulates NF-κB Transcriptional Activity**

Anissa E. Buckner and Richard D. Dix, Department of Ophthalmology, Jones Eye Institute, University of Arkansas for Medical Sciences, Little Rock, AR, USA

Little information is presently available regarding interaction of human cytomegalovirus (HCMV) at the molecular level with various cell types of the retina during AIDS-related CMV retinitis. Previous work has shown that soon after HCMV infection of many non-retinal cell types, transcription factors such as NF-κB, AP-1 and Sp1 are induced to regulate viral gene expression. Moreover, NF-κB plays an important role in immune and inflammatory responses associated with many viral genes. We therefore explored the effect of HCMV infection on the expression of NF-κB in human ARPE-19 cells, a cell line with properties similar to primary cultures of human retinal pigment epithelial (RPE) cells of the eye.

ARPE-19 cells transfected by cationic lipid-mediated NF-κB luciferase DNA (pNF-κB-Luc) alone exhibited promoter activity at a level of ~30,000 relative light units (RLU). In comparison, cells transfected with pNF-κB-Luc in the presence of HCMV infection showed a 15-fold decrease in promoter activity. As expected, a positive control for a mitogen-activated protein kinase (pFC-MEKK) showed a 2-fold increase in luciferase activity.

We conclude that this observation supports the finding of Cinatl et al. (2001) that HCMV circumvents NF-κB dependence in RPE cells. Thus, ARPE-19 cells can be used instead of primary RPE cells to study transcription factors involved in viral- host gene regulation.

Supported by NIH grant EY10568, Research to Prevent Blindness, and the Pat & Willard Walker Eye Research Center

112 **IKK-beta specific inhibitor PS1145 down-regulates NF-kappaB activity and induces apoptosis in prostate carcinoma cell lines.**

A. Yemelyanov[1], A. Gasparian[2], L. Dang[3], J. Pierce[3], I. Budunova[1].
[1]-Northwestern University, Chicago, IL, [2]- National Cancer Research Center, Moscow, Russia, [3]- Millenium Pharmaceuticals Inc., Cambridge, MA

Prostate cancer (PC) is the second leading cause of death among cancers in men. One of the contributing factors to high mortality rate from PC is the extreme resistance of malignant prostate cells to apoptosis induced by radio- and chemotherapy. Thus, the specific induction of apoptosis in PC cells could play a strategic role for PC treatment. One of the central mechanisms protecting cells from apoptotic death is mediated by NF-kappaB factors that control the expression of numerous anti-apoptotic genes. We and others showed previously that NF-kappaB transcription factor was constitutively active in PC cell lines and in human prostate tumors due to the up-regulated activity of IkappaB-kinases (IKK), mostly IKK-beta. In this work we investigated effect of PS-1145, a specific IKK-beta inhibitor, on constitutive and inducible NF-kappaB activity in human PC cell lines (PC-3 and DU-145) by Western blot analyses of IkBalpha phosphorylation & degradation, p65 nuclear translocation, EMSA, x5.kappaB-Luciferase gene reporter assay, and by Northern blot analysis of expression of endogenous kappaB-responsive genes. Our studies revealed that PS1145 at the dose range 5-20 μM efficiently inhibited both basal and TNF-alpha or LPS-induced NF-kappaB activity in PC cells. Furthermore, as PC3 and DU145 cells are known to be resistant to TNF-alpha-induced apoptosis, partially due to the constitutively active NF-kappaB, we found that PS1145 significantly sensitized PC cell lines to TNF-alpha induced apoptosis. Consistent with that finding, we observed the elevated PARP cleavage and caspase 3/7 activation when cells exposed to TNF-alpha were pretreated with PS1145. Currently we are evaluating the expression of kappaB-responsive genes as well as PC gene markers in prostate cell lines upon PS1145 treatment.

353 **Importance of NFκB in neointimal formation after balloon injury in porcine coronary artery**

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Percutaneus transluminal coronary angioplasty (PTCA) is a modern treatment regime of coronary diseases. However, restenosis after PTCA is a serious problem. Although mechanisms of restenosis remain unclear, the proliferation and migration of vascular smooth muscle cells (VSMC), and local inflammation are generally considered to play an important role in the progression of atherosclerotic plaque and subsequent restenosis of coronary arteries. The transcription factor, nuclear factor-kappa B (NFκB), plays a pivotal role in the transactivation of cytokine and adhesion molecule genes. We hypothesized that inhibition of NFκB activation may lead to prevent restenosis after balloon injury. To prove this hypothesis in this study we examined the effect of cerivastatin, one of the HMG-CoA reductase inhibitors, that is known to inhibit NFκB activity and NFκB decoy oligodeoxynucleotides (ODN) on neointimal formation after balloon injury in porcine. Vehicle (n=8) or cerivastatin (n=8) was orally administered at 1mg/kg/day from 7 days before lasting up to 4 weeks after balloon injury. We also transfected NFκB and scrambled decoy ODN into the balloon-injured artery using a hydrogel catheter. After 4 weeks, the histological staining demonstrated a significant inhibition of neointimal formation by cerivastatin and NFκB decoy ODN ($p<0.01$). In addition, the impaired response of endothelium to bradykinin in balloon-injured vessels was significantly improved by treatment with cerivastatin and also NFκB decoy ODN ($p<0.05$). In these analysis vehicle or scrambled decoy ODN had no effects. Overall, the present study indicates that inhibition of NFκB activity by cerivastatin or decoy ODN has a direct inhibitory effect on neointimal formation and improvement of endothelial dysfunction. These data suggest the importance of NFκB in the restenosis and also that NFκB decoy ODN approach could be an effective strategy for restenosis after coronary angioplasty.
1)Keita Yamasaki, 2)+81-6-6879-3401, 3)A4

354 **Stimulus-specific Induction of IκB-ζ, a Novel Regulator of NF-κB**

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We have recently identified a novel nuclear protein, IκB-ζ. IκB-ζ preferentially associates with the NF-κB subunit p50 rather than p65, through which it regulates the activity of NF-κB. IκB-ζ is hardly detectable in resting cells such as macrophages and fibroblasts and is induced by various microbial components including lipopolysaccharide (LPS) and peptidoglycan, or by the inflammatory cytokine interleukin (IL)-1β. Tumor necrosis factor (TNF)-α, however, dose not elicit the induction of IκB-ζ although it induces the activation of NF-κB or p38 MAP kinase as LPS or IL-1β does. Analyses on the promoter and studies using various inhibitors indicated that NF-κB was essential for the induction of IκB-ζ. Transcriptional activation, however, does not appear to account for the stimulus-specific induction, since the nuclear run-on assay indicated that LPS, TNF-α, or IL-1β up-regulated the transcription of IκB-ζ to the similar extent. Then, we established NIH/3T3 cell lines constitutively expressing the full-length IκB-ζ mRNA under the control of chicken β-actin promoter, and evaluated the stability of the mRNA. The decay of IκB-ζ mRNA was specifically delayed when the cells were stimulated with LPS or IL-1β, but not with TNF-α. These results indicate that the specific induction of IκB-ζ is regulated at the level of the stability of mRNA, which is up-regulated by LPS or IL-1β. The specific induction of IκB-ζ might be critically involved in the regulation of NF-κB-mediated transcription.

355 **Possible role of IKKi in the constitutive activation of NF-kappaB in prostate carcinoma cells**

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Our recent data and data by others indicate that NF-kappaB is constitutively activated in androgen-independent prostate carcinoma (PC) cells and prostate tumors, and that NF-kappaB activation promotes PC cells' tumorigenicity, invasiveness and resistance to apoptosis. The important step in NF-kappaB activation is the phosphorylation of IkappaB inhibitor proteins by IKK kinases: IKKalpha, IKKbeta and IKK-related inducible kinase IKKi. IKKi is the only IKK whose activity is regulated by its expression. We found that IKKalpha and IKKbeta were uniformly expressed in primary prostate cells and PC cell lines. On contrast, IKKi was strongly expressed only in androgen-independent PC cells (PC3 and DU145) with high level of constitutively active NF-kappaB but not in androgen-dependent PC cell lines (LNCaP and MDA PCa 2b) and primary prostate epithelial cells. Immunostaining also revealed that IKKi was expressed in human prostate carcinomas. Treatment of PC cells with NF-kappaB inducers such as IL-1 alpha and TNF-alpha resulted in a rapid induction of IKKi. Consistent with this, down-regulation of NF-kappaB activity by proteasome inhibitor MG132 attenuated induction of IKKi expression by NF-kappaB inducers. Transient transfection of different PC cell lines with IKKi w.t. resulted in activation of kappaB.Luciferase reporter, whereas IKKi dominant negative (d.n.) mutant K38A suppressed basal NF-kappaB activity in PC cells. These data provide experimental evidence that IKKi could be involved in the regulation of NF-kappaB activity in PC cells through a positive feedback loop. Supported by DOD Prostate Cancer Research Program DAMD 17-03-1-0522.

356 **Stable Transfection Of A Mutated I Kappa B Alpha Into HNSCCCal27 Cells Leads To Differential Sensitivity To 5-Fuourouacil and Cis-Platinum**

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Cal27 is an established cancer cell line derived from a Head and Neck squamous carcinoma (HNSCC). This tumor cell line is tumorigenic in nude mice and has been show to be resistant to a variety of chemotherapeutic reagents, including Cis-platinum and 5- Fuourouacil (5-FU) which are used to treat HNSCC. We have found that this cell line exhibits a high level of NF-kappaB DNA binding activity and moderate NF-kappa B-Luciferase activity. Un-transfected Cal27 carries detectable levels of I kappa B alpha. One of the stably transfected clones showed one-fold increase of I kappa B alpha protein level and about 75% decrease of NF-kappa B luciferase activity compared to un-transfected Cal27 cells. This decreased NF-kappa B activity doesn't inhibit cell survival and proliferation in these transfected cells, but does sensitize these cells to 5-FU 10,000 fold. Only about 20% increase in inhibition response to Cis-Platnium compared to un-trasfected cells. Similar, but stronger, inhibitory effects by 5-FU were also observed in a sub-population of a pool of the transfected cells with lower increased I kappa B alpha protein level. Our result suggest that NF-kappa B activity may play a greater role in resistance to 5-FU than Cis-Platinum.

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4572 Effect of IKK-beta specific inhibitor PS1145 on NF-kappaB activity and apoptosis in prostate carcinoma cell lines.

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Prostate cancer (PC) is the second leading cause of death among cancers in men. One of the contributing factors to high mortality rate from PC is the extreme resistance of malignant prostate cells to apoptosis induced by radio- and chemotherapy. Thus, the specific induction of apoptosis in PC cells could play a strategic role for PC treatment. One of the central mechanisms protecting cells from apoptotic death is mediated by NF-kappaB factors that control the expression of numerous anti-apoptotic genes. We and others showed previously that NF-kappaB transcription factor was constitutively active in PC cell lines and in human prostate tumors due to the up-regulated activity of IkappaB-kinases (IKK), mostly IKK-beta. In this work we investigated effect of a novel highly specific IKK-beta inhibitor PS1145 on constitutive and inducible NF-kappaB activity in human cell lines PC-3 and DU145 using Luciferase Assay with x5.kappaB-Luciferase reporter, EMSA, Northern blot analysis of expression of endogenous kappaB-responsive genes, Western blot analysis of IkappaBalpha phosphorylation, degradation and p65 nuclear translocation. Our studies revealed that PS1145 at the dose range 5-20 μM efficiently inhibited both basal and induced by either TNF-alpha or LPS NF-kappaB activity in PC cells. PC3 and DU145 cells are known to be resistant to TNF-alpha-induced apoptosis partially due to the constitutively active NF-kappaB. We found that PS1145 significantly sensitized PC cell lines to TNF-alpha induced apoptosis. We observed the elevated PARP cleavage and caspase 3/7 activation when cells exposed to TNF-alpha were pretreated with PS1145. Currently we are evaluating the expression of kappaB-responsive genes as well as PC gene markers in prostate cells upon PS1145 treatment in vitro and in vivo. Supported by DOD prostate cancer research grants DAMD17-01-1-0015 and DAMD17-03-1-0522.

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